

The cytotoxic lipid peroxidation product, 4-hydroxy-2-nonenal, specifically inhibits decarboxylating dehydrogenases in the matrix of plant mitochondria

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Abstract 4-Hydroxy-2-nonenal (HNE), a cytotoxic product of lipid peroxidation, inhibits O₂ consumption by potato tuber mitochondria. 2-Oxoglutarate dehydrogenase (OGDC), pyruvate dehydrogenase complex (PDC) (both 80% inhibited) and NAD-malic enzyme (50% inhibited) are its major targets. Mitochondrial proteins identified by reaction with antibodies raised to lipoic acid lost this antigenicity following HNE treatment. These proteins were identified as acetyltransferases of PDC (78 kDa and 55 kDa), succinyltransferases of OGDC (50 kDa and 48 kDa) and glycine decarboxylase H protein (17 kDa). The significance of the effect of these inhibitions on the impact of lipid peroxidation and plant respiratory functions is discussed. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: 4-Hydroxy-2-nonenal; Lipid peroxidation; Pyruvate; 2-Oxoglutarate

1. Introduction

The electron transport chains of chloroplast and mitochondria are major sites of active oxygen species (AOS) formation in plants [1]. Antioxidant systems found in both these subcellular compartments scavenge AOS and preserve the integrity and function of these organelles [2,3]. A range of biotic and abiotic stresses raise AOS levels in plants due to perturbations of metabolism and the generation of AOS in defence responses [4]. Such accumulation of AOS in plants results in a wide variety of deleterious effects through oxidation reactions involving proteins, lipids and nucleic acids. Research on plant oxidative stress to date has been centred on investigation of sensitive reactions in the chloroplast and antioxidant systems present in this organelle, while our understanding of the impact of oxidative stress on plant mitochondrial functions is limited.

Changes in the abundance and/or activities of a variety of plant mitochondrial proteins have been linked with response to AOS. Synthesis of the alternative oxidase was induced by H₂O₂ treatment or addition of respiratory inhibitors that raised mitochondria AOS production [5]. Further, the role of this enzyme in alleviating AOS production from the plant respiratory chain has been established [6]. Oxidative stress of tomato cell culture led to induction of a 22 kDa mitochon-

drial heat shock protein [7] and the expression of the mitochondrial phosphate carrier was reported to be induced by ozone treatment of lettuce [8]. The TCA cycle enzyme, aconitase, is also known to be sensitive to irreversible inhibition by H₂O₂ in potato mitochondria [9].

Polyunsaturated fatty acids of membrane lipids are highly susceptible to peroxidation by AOS to yield a variety of aldehydes, alkenals and hydroxyalkenals including the cytotoxic compounds malonaldehyde and 4-hydroxy-2-nonenal (HNE). These secondary endproducts are often collectively measured in studies of lipid peroxidation by the thiobarbituric acid-reactive substances assay [10]. Interest in the effects of these compounds in mammals, especially HNE, was stimulated by studies showing cytotoxicity due to the rapid reaction at neutral pH with sulphhydryl groups of cysteine via Michael addition [11]. A number of stress conditions, including cardiac reperfusion injury, increase AOS production, membrane peroxidation and decrease respiratory rate in mammals [12]. HNE has now been shown to directly inhibit respiration of isolated mammalian mitochondria oxidising glutamate and the effect has been pinpointed to 2-oxo acid dehydrogenases [13]. Interestingly, inhibition of these enzymes requires HNE to be present during catalysis by the enzyme and has been shown to involve reaction of HNE with the reduced form of the lipoic acid moiety at the enzyme active site [14]. A role for mitochondrial thioredoxins in modulating the redox status of lipoic acid moieties in 2-oxo acid dehydrogenases has been highlighted [15] and this mechanism may be a factor in understanding the stress response of mammalian mitochondria to damage from lipid peroxidation products.

Lipid peroxidation in plants has been known for many years, but definitive evidence of the pathways involved and the identification of HNE production in plants has only been described recently [16]. Here we show that HNE rapidly inhibits respiration of isolated plant mitochondria and four sites of action have been identified as the pyruvate dehydrogenase complex (PDC), the 2-oxoglutarate dehydrogenase complex (OGDC), NAD-dependent malic enzyme (NAD-ME) and the glycine decarboxylase complex (GDC).

2. Materials and methods

Potato tubers (*Solanum tuberosum* cv. Romano) were purchased locally and 5–10 kg used for isolation of Percoll gradient purified mitochondria according to Millar et al. [17]. PDC and OGDC were purified from isolated mitochondria by centrifugation and PEG precipitation protocols according to Millar et al. [17,18].

O₂ consumption was measured in an O₂ electrode (Hansatech, UK)

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in 1 ml of reaction medium containing 0.3 M mannitol, 10 mM TES-KOH pH 7.5, 5 mM KH_2PO_4 , 10 mM NaCl, 2 mM MgSO_4 and 0.1% (w/v) bovine serum albumin (BSA). Pyruvate (5 mM), malate (0.5 mM), 2-oxoglutarate (5 mM), succinate (10 mM), NADH (1 mM), NAD (0.5 mM), TPP (0.05 mM) and ADP (0.1–1 mM) were added as indicated. Activities of various enzymes were measured according to standard protocols: OGDC [18], PDC [17], NAD-ME [19], cytochrome *c* oxidase [20], citrate synthase [21], aconitase and fumarase [22], NAD-dependent isocitrate dehydrogenase [23] and NADH:ubiquinone oxidoreductase as NADH-dependent FeCN reduction [24]. Malate dehydrogenase was assayed as OAA-dependent NADH oxidation in 10 mM OAA, 0.2 mM NADH, 10 mM MgCl_2 and 50 mM TES-KOH pH 7.2. The component enzymes of mPDC, pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2) and dihydrolipoamide dehydrogenase (E3) were also measured as outlined previously [25]. Dithio-(bis)nitrobenzoic acid (DTNB) assays were performed according to Humphries and Szewda [14]. All enzyme activities were measured at 25°C. Protein concentrations were determined by the method of Bradford [26] using BSA as standard.

Total mitochondrial proteins and the subunits of PDC and OGDC preparations were separated by electrophoresis under denaturing, reducing conditions on 0.1% (w/v) sodium dodecyl sulphate (SDS)–12% (w/v) polyacrylamide gels according to Laemmli [27]. For immunoreaction experiments, proteins were electroblotted from SDS–PAGE gels onto nitrocellulose membranes and blocked in 5% (w/v) casein. 1/15 000 dilution of the anti-lipoic acid serum [14], 1/1000 dilution of the anti-H protein of GDC serum and 1/1000 dilution of the anti-acyl carrier protein (ACP) serum were used as primary antibodies. Chemiluminescence was used for detection of horseradish peroxidase-conjugated secondary antibodies.

3. Results and discussion

3.1. Effect of HNE on plant mitochondrial respiration

In the light of the significant inhibitory effect of HNE on mammalian respiratory function [13,14], we have investigated the effect of this lipid peroxidation product on the function of isolated plant mitochondria from potato tuber. Isolated potato mitochondria were incubated for 5 min in the presence of ADP, cofactors and a substrate or substrates that can be oxidised by the TCA cycle, in the presence or absence of 200 μM HNE. Following addition of 10 mM cysteine to quench remaining HNE, O_2 consumption in the presence of the incubation substrate was recorded using an O_2 electrode (Table 1). Succinate-dependent respiratory rate was not significantly affected by HNE treatment, while pyruvate+malate- and 2-oxoglutarate-dependent respiration were inhibited by 40% and 80%, respectively, following the HNE treatment. These results imply that HNE is not primarily acting on the plant mitochondrial electron transport chain, which is largely common to the pathway of O_2 consumption used by all three substrates, but rather on site(s) in the oxidation of TCA cycle substrates leading to reduction of the dinucleotide pool in the matrix.

3.2. Effect of HNE on mitochondrial enzyme maximal activities

To further investigate the sites of action of HNE on potato tuber mitochondria, the effects of this compound were tested on the maximal activity of known targets from mammalian systems and a range of other plant mitochondrial enzymes. Isolated potato tuber mitochondria were incubated for 5 min in the presence of 2-oxoglutarate, malate, pyruvate, cofactors and ADP, with or without 200 μM HNE. Reaction was stopped by addition of cysteine to 10 mM. Samples were then used to measure the maximal activities of enzymes (Table 2). Electron transport components involved in the transfer of electrons from the TCA cycle to O_2 were not significantly

Table 1

Effect of HNE on O_2 consumption rate of isolated potato mitochondria utilising different substrates

Substrate	O_2 consumption (+ADP) (nmol O_2 /min/mg protein)		
	control	+HNE	(control %)
Succinate	280 \pm 20	265 \pm 15	(95%)
Pyruvate+malate	200 \pm 15	120 \pm 20	(60%)
2-Oxoglutarate	285 \pm 32	57 \pm 5	(20%)

inhibited (cytochrome *c* oxidase, NADH dehydrogenases and succinate dehydrogenase). A number of TCA cycle enzymes were also not affected by HNE treatment (citrate synthase, aconitase, fumarase, isocitrate dehydrogenase and malate dehydrogenase). Of the three enzymes that were affected, the two 2-oxo acid dehydrogenases (PDC and OGDC) were inhibited more than 80% while another decarboxylating dehydrogenase (NAD-ME) was inhibited by 50%.

3.3. Lipoic acid moieties in plant mitochondrial proteins

Lipoic acid has been identified as a key target of HNE action and is found as a covalently bound moiety in subunits of both PDC and OGDC. Antibodies raised against the unmodified form of lipoic acid [14] were used here to detect lipoic acid moieties in plant mitochondrial proteins separated by SDS–PAGE (Fig. 1). Immunoblots consistently showed the presence of five protein bands reacting with the lipoic acid antibodies with apparent molecular masses of 78 kDa, 55 kDa, 50 kDa, 48 kDa and 17 kDa. Separation of the 50 and 48 kDa proteins was sometimes difficult to observe and antibody reaction then showed a broad band at 48–50 kDa (Fig. 1). Following the HNE treatment described above, all five proteins had greatly reduced reactivity with the lipoic acid antibody. Humphries and Szewda [14] showed that this antibody failed to recognise lipoic acid moieties modified as HNE–Michael adducts. The results shown in Fig. 1 are thus

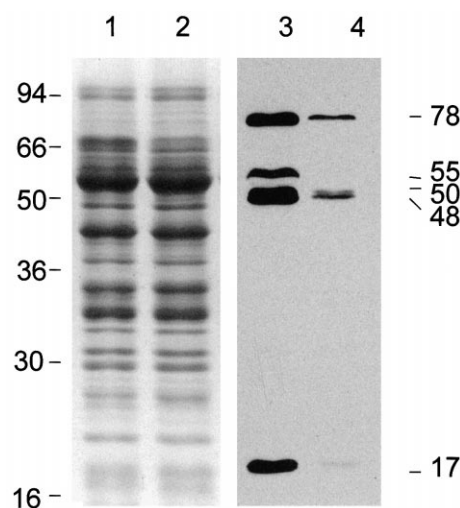


Fig. 1. Loss of reaction between plant mitochondrial proteins and anti-lipoic acid antibodies following treatment with HNE. Coomassie blue-stained protein from SDS–PAGE separation of proteins from (1) control mitochondria, (2) 200 μM HNE-treated mitochondria. Immuno-reaction of (3) control mitochondria and (4) 200 μM HNE-treated mitochondria with antibodies raised to protein-bound lipoic acid. Numbers are apparent molecular masses in kDa.

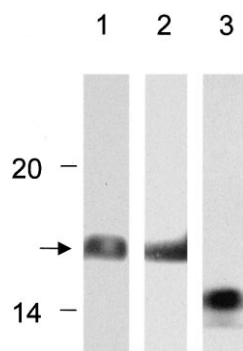


Fig. 2. Identification of the 17 kDa lipoic acid-containing protein of potato tuber mitochondria as the H protein of GDC. Reaction of (1) anti-lipoic acid antibodies, (2) anti-H protein of GDC antibodies and (3) anti-ACP antibodies, with SDS-PAGE-separated potato mitochondrial proteins. Numbers are apparent molecular masses in kDa, arrow indicates 17 kDa lipoic acid-containing H protein.

consistent with a loss of antigenicity of this moiety through HNE modification. The apparent molecular masses of the lipoic acid-containing protein bands are identical to those of four proteins in potato mitochondria known to contain covalently bound lipoic acid moieties. Proteins of 78 kDa and 55 kDa are the acetyltransferases of PDC [25], the succinyltransferases of OGDC are 48–50 kDa [18]. The 17 kDa immuno-reactive protein was tentatively assigned as either the 14–18 kDa ACP which is involved in lipoic acid addition to proteins in mitochondria [28] or as the approximately 16 kDa H protein of the GDC [29]. Identification of the 17 kDa protein as the H protein was confirmed by cross-reaction of

this band with antibodies raised to purified H protein of GDC isolated from pea mitochondria (Fig. 2, lanes 1 and 2). Antibodies raised to the ACP from *Arabidopsis* [30] reacted with a smaller band in potato mitochondria (Fig. 2, lane 3). No significant rate of glycine-dependent O_2 consumption could be recorded by potato tuber mitochondria (data not shown), thus we were unable to confirm whether GDC operation was inhibited by HNE.

3.4. Effect of HNE on purified PDC and OGDC

We have recently purified PDC and OGDC to homogeneity from potato tuber mitochondria [17,18,25]. Incubation of purified OGDC and PDC with 200 μ M HNE for 0, 5 and 15 min in the presence or absence of the complex's substrate, and subsequent assay of complex activities, showed that function was only significantly inhibited by HNE during catalytic turnover of the enzyme complexes (Fig. 3A). Subsequent analysis of the immuno-reactivity of the acetyltransferase and succinyltransferase subunits with lipoic acid antibodies showed a decrease in lipoic acid antigenicity that correlated with the loss of enzymatic function (Fig. 3B). No effect of HNE was found on pyruvate dehydrogenase (E1) activity measured as pyruvate:FeCN reduction, or dihydrolipoamide dehydrogenase (E3) activity measured as dihydrolipoamide:NAD reduction (data not shown). This suggests the effect of HNE is limited to a site on the E2 subunits of these enzymes.

Dihydrolipoamide acetyltransferase (E2) activity measured as reduction of exogenously added lipoamide by acetylCoA was not affected by HNE (data not shown). However, the rate of DTNB reactivity of PDC and OGDC samples, which is a measure of the ability of DTNB to bind to reduced lipoic

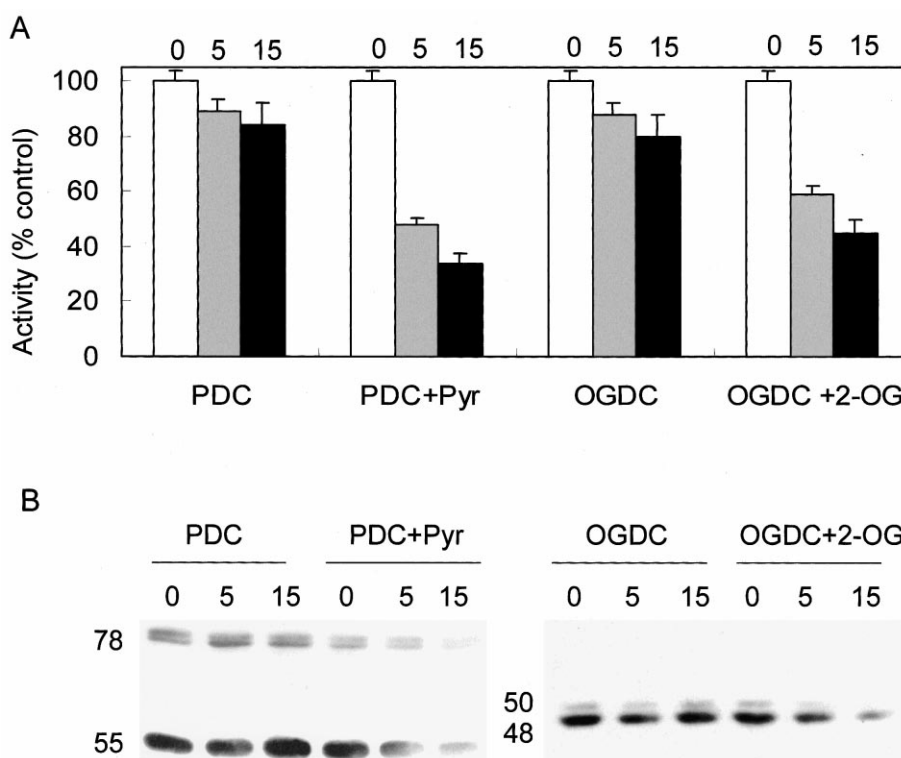


Fig. 3. Substrate-dependent inhibition of purified PDC and OGDC and loss of reaction between acetyltransferase and succinyltransferase subunits and anti-lipoic acid antibodies. A: Rates of purified PDC and OGDC activities after 0–15 min in the presence of 200 μ M HNE and all cofactors required for activity, with or without addition of pyruvate (Pyr) or 2-oxoglutarate (2-OG). B: Immuno-reaction of SDS-PAGE-separated, nitrocellulose-blotted PDC and OGDC proteins from the experiment in (A) with antibodies raised to protein-bound lipoic acid.

Table 2

Effect of HNE treatment on selected maximal enzyme activities of isolated potato mitochondria

	Control (nmol/min/mg protein)	+HNE	(Control %)
NADH-FeCN	6 750 ± 160	7 080 ± 130	(105%)
SDH	340 ± 35	380 ± 15	(104%)
COX	1 360 ± 90	1 375 ± 65	(101%)
PDC	190 ± 15	38 ± 12	(20%) ^a
MDH	30 100 ± 900	29 500 ± 1 500	(98%)
CS	405 ± 30	425 ± 20	(105%)
Aconitase	270 ± 15	273 ± 25	(101%)
ICDH	376 ± 46	335 ± 26	(90%)
OGDC	275 ± 20	52 ± 9	(19%) ^a
Fumarase	180 ± 5	188 ± 12	(104%)
NAD-ME	730 ± 40	350 ± 20	(48%) ^a

Enzymes were assayed according to Section 2.

^aSignificantly different from control ($P < 0.05$).

acid, was decreased by HNE. After HNE treatment for 15 min in the presence of substrate, DTNB reactivity decreased by $80 \pm 10\%$ and $70 \pm 8\%$ ($n = 3$) for PDC and OGDC samples, respectively. These data imply that the sole effect of HNE on these complexes appears to be the modification of lipoic acid moieties, disabling them from providing acetyl groups to CoA transferase sites on E2 and from transferring electrons to E3.

3.5. Lipid peroxidation and plant respiratory function

While various measurements of plant mitochondrial lipid peroxidation have been undertaken [31–33], no reports have attempted to show that mitochondrial function changes as a result of this damage. Despite the abundance of research on mechanisms for detoxifying AOS, there are still no confirmed biochemical pathways for the detoxifying of cytotoxic lipid peroxidation products in plants. Understanding of such pathways will be essential to assess the impact of lipid peroxidation on HNE-sensitive enzymes in plants such as those described here.

NAD-ME is found ubiquitously in plant mitochondria where it is thought, together with PEP carboxylase in the cytosol, to enable the anaplerotic function of the TCA cycle to supply metabolic intermediates for biosynthesis. The identification of NAD-ME as an HNE-sensitive enzyme in plant mitochondria (Table 2) may be due to the presence of susceptible cysteine residues in this enzyme, as HNE is known to modify cysteine residues [14]. Assays for NAD-ME routinely include dithiothreitol to maintain activity and mercurials are known inhibitors of this enzyme, suggesting the presence of susceptible, essential cysteine residues at its active site.

Losses of PDC and OGDC activities in plant mitochondria will slow complete TCA cycle activity. Oxidation of succinate or malate to oxaloacetate could be maintained providing oxaloacetate is removed from the mitochondrial matrix to prevent feedback inhibition of MDH. We have recently postulated, on the basis of analysis of plants expressing anti-sense RNA constructs of TCA cycle enzyme subunits in potato, that PDC has a high control coefficient over respiratory flux in plants [34]. The susceptibility of mPDC to HNE may thus suggest that the enzyme complex represents a prime target for oxidative stress-induced modification of plant respiratory function. Additionally, the catalytic activity of PDC E1 can be controlled by phosphorylation (inactivating) and dephosphorylation (activating) of the E1 α subunit. As the inhibition of PDC by HNE is dependent on enzyme catalytic turnover (Fig.

2), this regulatory mechanism has the potential to halt operation of the complex in the presence of substrate to prevent irreversible loss of PDC activity. Interestingly, phosphorylation of PDC is known to occur following exposure of plants to light [35] and this suggests a decrease in TCA cycle operation during periods of photosynthesis during which AOS production in the cell will be high.

HNE is also likely to modify the lipoic acid moieties of the H protein of the GDC. The prominent role of this enzyme in the photo-respiratory cycle in plants [36] highlights the importance of understanding the process of lipid peroxidation in plant mitochondria and the mechanisms of repair. The recent identification of pathways for lipoic acid attachment to acyl-transferase subunits in mitochondria [28] and the discovery of a pathway for de novo lipoic acid synthesis in plant mitochondria from photosynthetic tissues [29] will provide the basis for tools to investigate such mechanisms.

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References

- [1] Elstner, E.F. (1991) in: Active Oxygen/Oxidative Stress and Plant Metabolism (Pell, E. and Steffen, K., Eds.), Vol. 6, pp. 13–25, American Society of Plant Physiologists, Rockville.
- [2] Noctor, G. and Foyer, C.H. (1998) *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 49, 249–279.
- [3] Jimenez, A., Hernandez, J.A., del Rio, L.A. and Sevilla, F. (1997) *Plant Physiol.* 114, 275–284.
- [4] Van Camp, W., Van Montagu, M. and Inze, D. (1998) *Trends Plant Sci.* 3, 330–334.
- [5] Vanlerberghe, G.C. and McIntosh, L. (1997) *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 48, 703–734.
- [6] Maxwell, D.P., Wang, Y. and McIntosh, L. (1999) *Proc. Natl. Acad. Sci. USA* 96, 8271–8276.
- [7] Banzet, N., Richaud, C., Deveaux, Y., Kazmaier, M., Gagnon, J. and Triantaphylides, C. (1998) *Plant J.* 13, 519–527.
- [8] Kiiskinen, M., Korhonen, M. and Kangasjarvi, J. (1997) *Plant Mol. Biol.* 35, 271–279.

- [9] Verniquet, F., Gallard, J., Neuburger, M. and Douce, R. (1991) *Biochem. J.* 276, 643–648.
- [10] Hodges, D.M., Delong, J.M., Forney, C.F. and Prange, R.K. (1999) *Planta* 207, 604–611.
- [11] Esterbauer, H., Schaur, R.J. and Zollner, H. (1991) *Free Radic. Biol. Med.* 11, 81–128.
- [12] Lucas, D.T. and Szveda, L.I. (1998) *Proc. Natl. Acad. Sci. USA* 95, 510–514.
- [13] Humphries, K.M., Yoo, Y. and Szveda, L.I. (1998) *Biochemistry* 37, 552–557.
- [14] Humphries, K.M. and Szveda, L.I. (1998) *Biochemistry* 37, 15835–15841.
- [15] Bunik, V., Follmann, H. and Bisswanger, H. (1997) *Biol. Chem.* 378, 1125–1130.
- [16] Takamura, H. and Gardner, H.W. (1996) *Biochim. Biophys. Acta* 1303, 83–91.
- [17] Millar, A.H., Knorpp, C., Leaver, C.J. and Hill, S.A. (1998) *Biochem. J.* 334, 571–576.
- [18] Millar, A.H., Hill, S.A. and Leaver, C.J. (1999) *Biochem. J.* 334, 571–576.
- [19] Day, D.A., Neuburger, M. and Douce, R. (1984) *Arch. Biochem. Biophys.* 231, 233–242.
- [20] Neuburger, M., Journet, E., Bligny, R., Carde, J. and Douce, R. (1982) *Arch. Biochem. Biophys.* 217, 312–323.
- [21] Stitt, M. (1984) *Methods Enzym. Anal.* 4, 353–358.
- [22] MacDougall, A.J. and Ap Rees, T. (1991) *J. Plant Physiol.* 137, 683–690.
- [23] Cox, G.F. (1969) *Methods Enzymol.* 13, 47–51.
- [24] Herz, U., Schroder, W., Liddell, A., Leaver, C.J., Brennicke, A. and Grohmann, L. (1994) *J. Biol. Chem.* 269, 2263–2269.
- [25] Millar, A.H., Leaver, C.J. and Hill, S.A. (1999) *Eur. J. Biochem.* 264, 973–981.
- [26] Bradford, M.M. (1976) *Anal. Biochem.* 72, 258–264.
- [27] Laemmli, U.K. (1970) *Nature* 227, 680–685.
- [28] Jordan, S.W. and Cronan Jr., J.E. (1997) *J. Biol. Chem.* 272, 17903–17906.
- [29] Gueguen, V., Macherel, D., Jaquinod, M., Douce, R. and Bourguignon, J. (2000) *J. Biol. Chem.* 275, 5016–5025.
- [30] Shintani, D.K. and Ohlrogge, J.B. (1994) *Plant Physiol.* 104, 1221–1229.
- [31] Vianello, A., Marci, F. and Bindoli, A. (1987) *Plant Cell Physiol.* 28, 1263–1269.
- [32] Vianello, A., Macri, F., Cavallini, L. and Bindoli, A. (1986) *J. Plant Physiol.* 125, 217–224.
- [33] De Santis, A., Landi, P. and Genchi, G. (1999) *Plant Physiol.* 119, 743–754.
- [34] Millar, A.H., Saeed, S., Jenner, H.L., Knorpp, C., Leaver, C.J. and Hill, S.A. (1998) in: *Plant Mitochondria: from Gene to Function* (Moller, I., Gardestrom, P., Glimelius, K. and Glaser, E., Eds.), pp. 551–557, Backhuys Publishers, Leiden.
- [35] Budde, R.J.A. and Randall, D.D. (1990) *Proc. Natl. Acad. Sci. USA* 87, 673–676.
- [36] Oliver, D.J. (1994) *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 45, 323–337.