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On the function of a K-type vitamin in plasma membranes of maize (*Zea mays* L.) roots

Sabine Lüthje and Michael Böttger

Institut für Allgemeine Botanik und Botanischer Garten, Universität Hamburg, Ohnhorststraße 18, D-22609 Hamburg, Germany.
E-Mail: S.Luthje@plantphysiology.de

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On the function of a K-type vitamin in plasma membranes of maize (*Zea mays L.*) roots

Sabine Lüthje[1] and Michael Böttger

Summary: Occurrence of a K-type vitamin was demonstrated in plasma membranes of maize (*Zea mays* L.) roots by HPLC analysis. Significant amounts of ubiquinone-10 were not found. NADH:HCF III oxidoreductase activity increased after preincubation with naphthoquinones in the presence of detergent, while ubiquinone-10 was without an effect on this activity. The function of vitamin K in plasma membrane-bound redox activity is discussed.

Key Words: naphthoquinone, vitamin K, plasma membrane redox system, *Zea mays* L., maize roots

Abbreviations: dicumarol = 3,3'-methylene-bis (4-hydroxycoumarin); DTT = dithiothreitrol = $C_4H_{10}O_2S_2$; GSH = glutathione, oxidized form; HCF III = hexacyanoferrate III = ferricyanide = $K_3[FeCN_6]$; Hepes = 2-[4-(2-hydroxyethyl)-1-piperazine]-ethanesulfonic acid; HPLC = High Pressure Liquid Chromatography; menadione = vitamin K_3 = 2-methyl-1,4-naphthoquinone = $C_{11}H_8O_2$; PEG 3350 = polyethylene glycol 3350; PVP = polyvinylpyrrolidone; ubiquinone-10 = coenzyme Q_{10} = ubiquinone-50 = $C_{59}H_{90}O_4$; vitamin K_1 = 2-methyl-3-phytyl-1,4-naphthoquinone = 3-phytylmenadione; $C_{31}H_{46}O_2$;

A. Introduction

Redox activities at the plasma membrane of plants were found in all species investigated so far (Crane et al., 1991). An appearingly transmembrane electron transport was demonstrated for maize (*Zea mays* L.) roots *in vivo* by application of membrane impermeant artificial electron acceptors, e.g. hexacyanoferrate (III) (HCF III) or iridate complexes (Döring et al., 1990). The reduction of the acceptor in the external medium is accompanied by changes in NAD(P)H (Qiu et al., 1985; Krüger & Böttger, 1988; Krüger, 1993) and GSH levels (Seidenberg et al., 1995), depolarization of the membrane and an increased acidification of the medium were observed for maize roots (Döring et al., 1990). The natural electron acceptor and the physiological function of this redox activity (so-called 'standard'-reductase) is still a matter of discussion (Crane et al., 1991; Bienfait & Lüttge, 1988).

[1] To whom correspondence should be addressed

The increase in proton secretion observed after application of an artifical electron acceptor has been discussed controversal (BARR, 1991). The H^+/e^- stoichiometry found for maize roots was between 0.5 and 1 after application of HCF III (FEDERICIO & GIARTOSIO, 1984; BÖTTGER & HILGENDORF, 1988; DÖRING et al., 1990). This ratio was explained by activation of the H^+-ATPase after depolarization of the membrane and cytoplasmic acidification (RUBINSTEIN & STERN, 1986; MARRÉ et al., 1988). The activation energy of redox-induced proton secretion is lower compared to that of the plasma membrane H^+-ATPase at least for maize roots (HILGENDORF & BÖTTGER, 1993), which is in contrast to this hypothesis. Moreover ATPase inhibitors like vanadate or DES are not able to inhibit proton secretion completely in the presence of HCF III (RUBINSTEIN & STERN, 1986; BÖTTGER & LÜTHEN, 1986; BOWN & CRAWFORD, 1988) and low concentrations of external HCF III or iridate complexes cause an inhibition of net-proton secretion (BÖTTGER & HILGENDORF, 1988; DÖRING et al., 1990).

The H^+/e^- stoichiometry found for HCF III reduction might also be explained by a quinone, which acts as a redox loop (LIN, 1984; MALMSTRÖM, 1988). A substance with ubiquinone-like absorption spectrum was isolated from maize root protoplasts (LIN, 1984), but mitochondrial contamination could not be excluded for this investigation. Ubiquinone found in plasma membranes of cauliflower (*Brassica oleracea* L.) inflorescences (MØLLER et al., 1988) was later assumed to be an artifact caused by phenolic compounds (ASKERLUND, 1990; MØLLER et al., 1991).

Although ubiquinone-10 was not detected in plant plasma membranes, increasing evidence was found for involvement of a quinone in plasma membrane-bound redox activities. (i) Quinone-dependent NADH oxidation was repeatedly demonstrated with isolated plasma membranes (BUCKHOUT & LUSTER, 1991). (ii) Transmembrane HCF III reductase activity was inhibited up to 50 % after destruction of cellular quinones by UV-B radiation and activity was restored by addition of vitamin K_1 (BARR et al., 1992). (iii) Vitamin K_3 (menadione) stimulated transmembrane HCF III reductase activity of maize roots *in vivo* (DÖRING et al., 1992a; LÜTHJE et al., 1992). (iv) Coumarins (vitamin K antagonist) caused an inhibition of transmembrane electron transport of intact maize roots (DÖRING et al., 1992a, 1992b; LÜTHJE et al., 1992). (v) Inhibition of NADH:HCF III oxidoreductase activity by dicumarol could be reversed by addition of quinones (LÜTHJE et al., 1994). (vi) A dicumarol-sensitive NADH dehydrogenase was isolated from plasma membranes of onion (*Allium cepa* L.) roots (SERRANO et al., 1994).

The aim of the present study was investigation of the occurrence of quinones in plasma membranes and their possible function for plasma membrane-bound redox activities.

B. Material and Methods

Chemicals: Dextran T500 was from Pharmacia (Freiburg, Germany). Polyethylene glycol 3350, NADH, DTT, PVP and quinones were obtained from Sigma (Deisendorf, Germany). Solvents, HPLC grade, were from CS Chromatographie Service (Langerwehe, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

Plant material and redox activity: Maize (*Zea mays* L., c.v Sil Anjou 18) roots (Saatenunion, Hannover, Germany) were prepared as described elsewhere (LÜTHJE et al., 1994). NADH:HCF III oxidoreductase activity of plasma membrane vesicles was measured as described by (LÜTHJE et al., 1994). Plasma membranes (30–50 µg protein/ml) were preincubated with quinones, solved in 2-propanol at 4°C. 0.025 % Triton X-100 was added after a 3 minute preincubation. The reaction was started by addition of 100 µM HCF III and 120 µM NADH. Reduction rates were calculated using an extinction coefficient of 1.0 mM cm^{-1} for HCF III.

Quinone analysis: In a second set of experiments lipid extratcs of plasma membranes were investigated by high pressure liquid chromatography (HPLC). Plasma membranes (5-10 mg protein) were washed with 1 mM EDTA as described recently (LÜTHJE et al., 1995). The pellet was resuspended in double-destilled water and aliquots were used for protein determination (BRADFORD, 1976). Lipids were extracted in n-heptan/2-propanol (2/1, v/v) under nitrogen. n-heptan phases were evaporated to dryness under nitrogen at 22°C and lipids were taken up in n-heptan (HPLC grade). Isocratic HPLC was performed on a Spectra Physics system (SP 8800 and Spectra Focus detector; Spectra Physics, Darmstadt-Kranichstein, Germany). 50 µl of the sample was injected on a Hypersil-ODS (C18) reversed phase column (5 µm, 250 × 4.6 mm I.D.), guard column was also Hypersil-ODS (10 µm, 60 × 4.6 mm, CS-Chromatographie Service, Langerwehe, Germany). Lipophilic compounds were separated with n-heptan/2-propanol (1/9, v/v) as solvent with a flow rate of 1 ml per minute. Elution was monitored at 245 nm using the high speed scanning mode. Data were analysed by Spectra Focus software (Spectra Physics, Darmstadt-Kranichstein, Germany). On-line spectra and quinone standards were used for identification of the peaks and calculation of the amounts by an extinction coefficient of 19 mM cm-^{1} for vitamin K (BARR & CRANE, 1971).

C. Results

Standards and lipid extracts were stable in n-heptan, while storage in ethanol causes destruction of the substances (data not shown). The HPLC method used in the present investigation was highly sensitive, limit of detection was 10 pmol for vitamin K_1.

HPLC elution profiles of plasma membranes and standards were shown in fig. 1. The peak at 2.7 minutes found for lipid extracts had the same retention time as the vitamin K_1 standard. On-line spectra of these peaks were characteristical for K-type

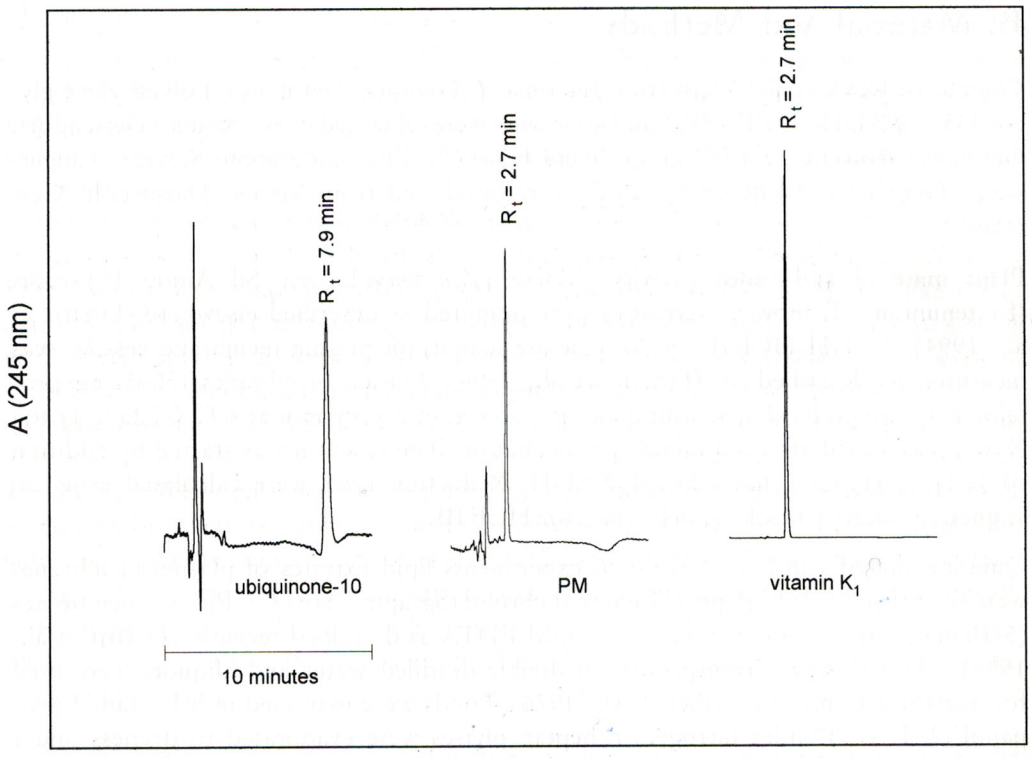


Fig. 1. HPLC elution profile of standards and n-heptan extracts of isolated plasma membranes at 245 nm. The peak at 2.7 minutes is identical to the vitamin K_1 standard, while ubiquinone-10 was not detected in plasma membranes.

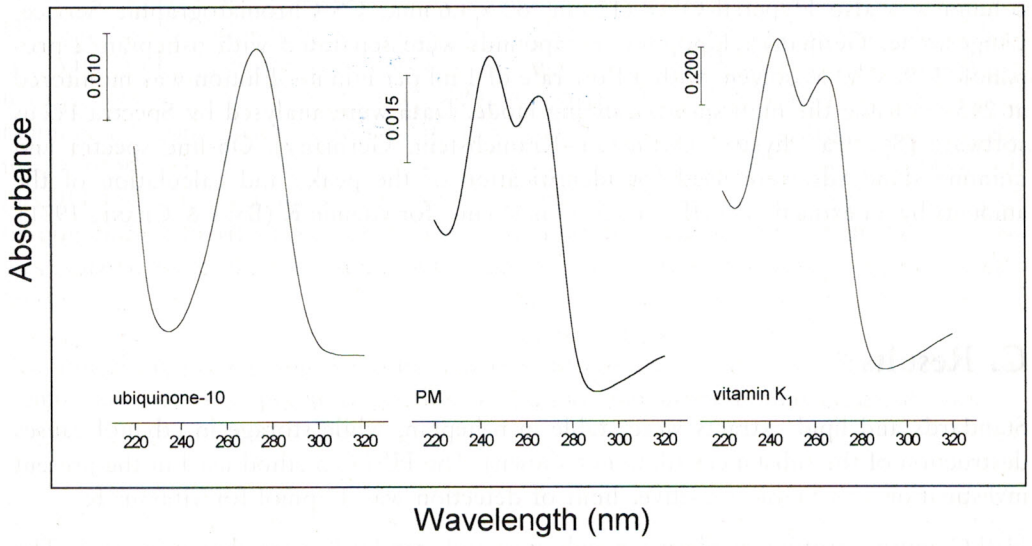


Fig. 2. On-line spectra of ubiquinone-10 and vitamin K_1 standards as well as n-heptan extracts of isolated plasma membranes measured at t_R = 2.7 minutes. Absorption maxima were found at 245 and 265 nm for the substance isolated from plasma membranes. These maxima were identical to authentic vitamin K_1.

vitamins (fig. 2). Absorption maxima found at 245 and 265 nm for plasma membrane extracts were identical to the vitamin K_1 standard. Calculations from the absorbance at 245 nm gave an amount of 9 nmol vitamin K (mg protein)$^{-1}$ for washed plasma membranes. Ubiquinone-10, which elutes with a retention time of 7.9 minutes was not found in plasma membrane preparations (fig. 1).

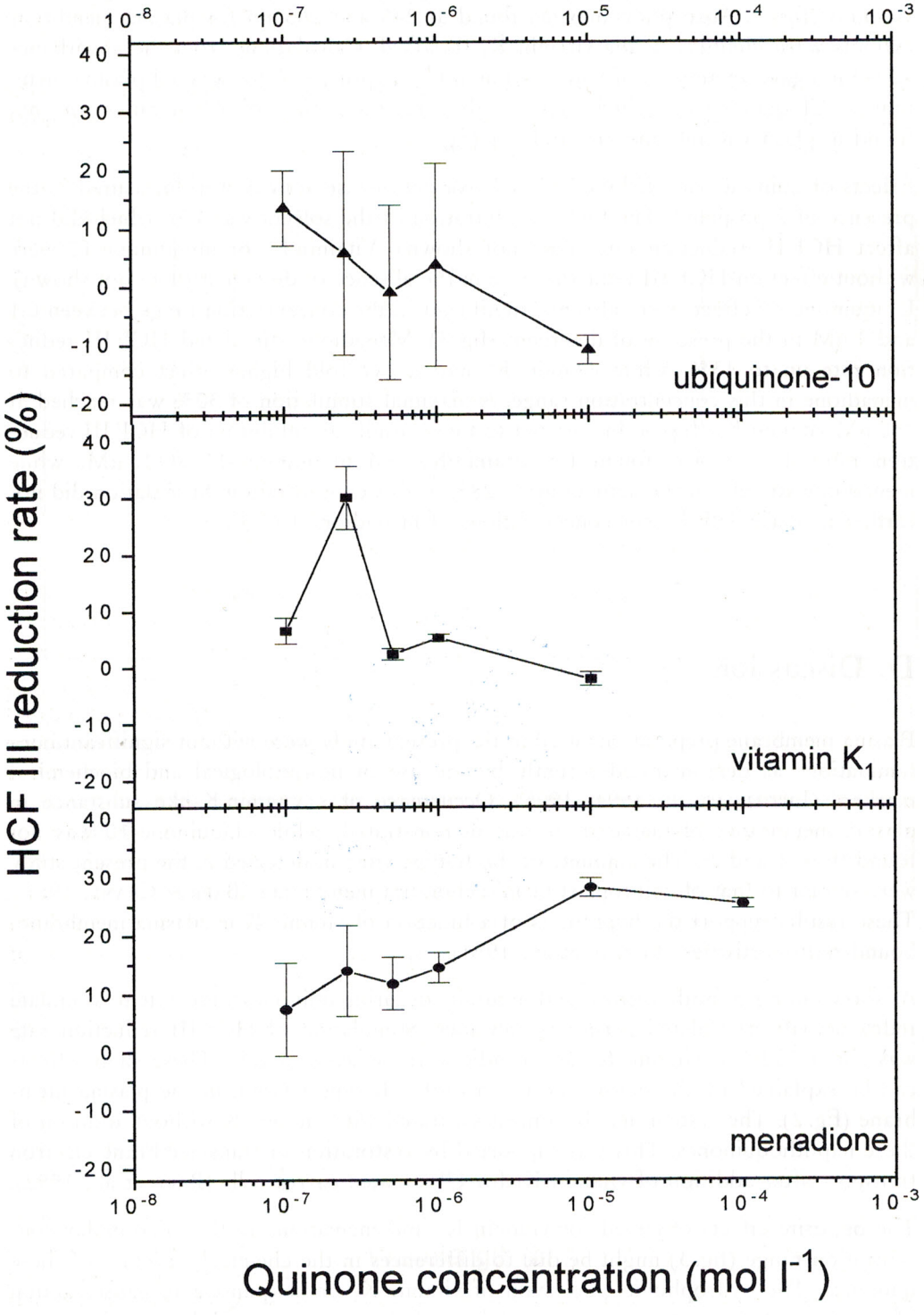
Effects of quinones on NADH:HCF III oxidoreductase activity were measured in the presence of 2-propanol. The final concentration of the solvent was 1 %, which did not affect HCF III reduction rate (data not shown). Vitamin K_1 or ubiquinone-10 were without effect on HCF III reduction rate in the absence of detergent (data not shown). Ubiquinone-10 effects were also not significant in the concentration range between 0.1 and 1 µM in the presence of detergent (fig. 3). Menadione stimulated HCF III reduction rate up to 13 %, while vitamin K_1 had a two-fold higher effect compared to menadione in this concentration range. A maximal stimulation of 30 % was reached at 250 nM vitamin K_1 (fig. 3). In contrast to these results an inhibition of HCF III reduction rate of 10 % was found for vitamin K_1 and ubiquinone-10 at 10 µM, while menadione stimulated the activity up to 28 % at this concentration. Stimulation did not further increase with higher concentrations of menadione (fig. 3).

D. Discussion

Plasma membrane preparations used in the present study were without significant contaminations as demonstrated recently by the use of morphological and biochemical markers (LÜTHJE et al., 1994, 1995). Occurrence of a vitamin K-like substance in plasma membranes of maize roots was demonstrated, while ubiquinone-10 was not found (figs. 1 and 2). The amounts of the K-type vitamin detected in the present study were similar to that of chloroplast or mitochondria membranes (BARR & CRANE, 1971). These results support the hypothesis of a function of vitamin K in plasma membrane-bound redox activities (LÜTHJE et al., 1994).

As shown in fig. 3, both artifical and naturally occuring quinones were able to stimulate redox activity of isolated plasma membranes. Stimulation of HCF III reduction rate was about 30 % for vitamin K_1 or menadione respectively (fig. 3). These slight effects can be explained by the natural occurrence of a K-type vitamin in the plasma membrane (fig. 2). The system may be almost saturated with vitamin K without addition of these naphthoquinones. This was supported by restoration of transmembrane electron transport after addition of vitamin K_1 for UV treated carrot cells (BARR et al., 1992).

The opposite effects observed for vitamin K_1 and menadione in the micromolar concentration range (fig. 3) might be due to differences in the chemical structure of these quinones. The hydrophobic side chain of vitamin K_1 or ubiquinone-10 causes action of this long chain quinones in the midplane of the membrane, while menadione was suggested to react also at the surface of the lipid bilayer (LENAZ et al., 1992; DÖRING


HCF III reduction rate (%)
Quinone concentration (mol l-1)
ubiquinone-10
vitamin K1
menadione

et al., 1992a; LÜTHJE et al., 1994). This hypothesis was supported by the K_M values found for duroquinone-dependent NADH oxidase activity (PUPILLO et al., 1986).

The slight inhibition of HCF III reduction rate found for long chain quinones in the micromolar concentration range (fig. 3) might be due to changes in membrane fluidity as demonstrated for mitochondria membranes incubated with ubiquinone-10 (SPISNI et al., 1978; FATO et al., 1984). On the other hand artifical quinones like menadione or duroquinone are well known as superoxide radical generators, while vitamin K_1 or ubiquinone-10 act as radical scavenger (CADENAS et al., 1977). Production of superoxide radicals, however, was observed for intact plants (AVERY'ANOFF, 1985) and at isolated plasma membrane vesicles at low pH in the presence of detergent (VIANELLO & MACRI, 1991). The duroquinone-dependent NADH oxidase isolated from zucchini microsomes produces H_2O_2 only in the presence of duroquinone (GUERRINI et al., 1987). These results suggest, that the K-type vitamin found in plasma membranes (fig. 2) might have a function in H_2O_2 production. Superoxide anion radicals and H_2O_2 production play a role in signal transduction (CRANE et al., 1991). Evidence for a function of K-type vitamins in growth control was found fourty years ago. Vitamin K_1 stimulated germination and elongation growth (HEMBERG, 1953; STOWE & OBREITER, 1962), while dicumarol inhibited these processes (MAYER, 1953).

The occurrence of a K-type vitamin in plasma membranes (fig. 2) indicated that the H^+-ATPase might be not the only activity transfering protons across this membrane. A quinone binds one proton per electron during reduction, which are released by its reoxidation. In fact, both inhibiton of net-proton secretion as well as stimulation can be observed after application of 0.2 mM menadione in dependence of the experimental setup (LÜTHJE et al., 1992). Simultaneously incubation of intact plants with menadione and HCF III results in an inhibition of HCF III reduction and proton secretion, while a stimulation of transmembrane HCF III reduction and net-proton secretion was observed after preincubation with menadione followed by intense rinsing. The later observation was explained by a menadione pool in the membrane (DÖRING et al., 1992, LÜTHJE et al., 1992), while menadione in the incubation medium can compete with HCF III for the electrons transfered across the lipid bilayer. Reduction of menadione by a trans-membrane electron transfer was demonstrated by depolarization of the membrane potential (BERNSTEIN et al., 1989; DÖRING et al., 1992).

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Fig. 3. Modulation of NADH:HCF III oxidoreductase activity by the natural isoprenoid quinones ubiquinone-10 and vitamin K_1 or by the artifical menadione (solved in 2-propanol) *in vitro*. Data presented were from one plasma membrane preparation but were reproducible with three independent preparations. Values given for HCF III reduction are average values of five experiments corrected by the rates of chemical control experiments, i.e. experiments without plasma membranes. Control was set to 0%.

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Address of the authors: Sabine LÜTHJE, Michael BÖTTGER, Institut für Allgemeine Botanik und Botanischer Garten, Universität Hamburg, Ohnhorststraße 18, D-22609 Hamburg, Germany. FAX +49-40-82282-254, E-mail fb4a044@phylu.physnet2.uni-hamburg.de