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RESEARCH PAPER

Transmembrane electron transport in sealed and NAD(P)H-loaded right-side-out plasma membrane vesicles isolated from maize (*Zea mays* L.) roots

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Abstract

Electron transport across plasma membranes has been observed *in vivo* in several plant species and tissues after the application of ferricyanide (hexacyanoferrate III, HCF III). In the present work, a transmembrane electron flow was demonstrated in sealed and NAD(P)H–loaded right-side-out (apoplastic-side-out) plasma membrane vesicles isolated from maize (*Zea mays* L.) roots. HCF III was reduced at a rate of up to 126 nmol min^{-1} mg^{-1} protein by NADPH-loaded vesicles, while reduction rates with NADH-loaded vesicles were several-fold lower. Coincident with the reduction of HCF III, NAD(P)H oxidation was observed inside the vesicles. The dependence of reduction on K^+ indicated an electrogenic transmembrane electron flow. Application of 100 μM calcium decreased HCF III reduction up to 66%, while preincubation with 200 μM warfarin or diphenylene iodonium inhibited transmembrane electron transport only weakly. Fe^{3+}-EDTA was not reduced significantly by NADPH-loaded plasma membrane vesicles, whereas XTT was reduced at a rate of 765 pmol min^{-1} mg^{-1} protein. The results suggested a major function for NADPH in transmembrane electron flow and were discussed in conjunction with *in vivo* experiments.

Key words: Calcium, diphenylene iodonium, NAD(P)H-loaded vesicles, plasma membrane, transmembrane electron transport, *Zea mays*.

Introduction

Reduction of artificial electron acceptors like ferricyanide (hexacyanoferrate III, HCF III) by several intact plant organs has been known since the early 1980s (Lüthje *et al.*, 1997; Bérczi and Møller, 2000). Besides a reduction of membrane impermeable electron acceptors, a depolarization of the plasma membrane and an increase in apoplastic acidification was observed. Based on the depolarization, a transmembrane electron flow has been postulated for this constitutive oxidoreductase (redox) activity, the so-called standard system. Further investigations indicated that NAD(P)H and oxygen were natural substrates, and ion uptake, radical scavenging, and/or cell defence were discussed as possible functions (Bienfait and Lüttge, 1988; Møller and Crane, 1990; Rubinstein and Luster, 1993; Lüthje *et al.*, 1997; Bérczi and Møller, 2000).

Besides this constitutive system, iron reductases have been described in dicotyledons and non-grass monocotyledons that reduce iron prior to uptake (Römheld and Marschner, 1986; Bienfait and Lüttge, 1988; Schmidt, 1999). In pea (*Pisum sativum*) a constitutive FRO1 (ferric reductase-oxidase) was identified and demonstrated to be induced under iron deficiency (Waters *et al.*, 2001). FRO2 represented a constitutive iron reductase activity in *Arabidopsis* which plays a key role in root iron uptake in response to iron deficiency (Robinson *et al.*, 1999; Vert *et al.*, 2001, 2003). Grasses, however, evolved a completely different iron uptake strategy. These strategy II plants excrete phytosiderophores and formed Fe^{3+}-chelates that are reabsorbed and reduced inside the cell (Bienfait,

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Abbreviations: BPDS, bathophenanthroline disulphonate; Brij 58, polyoxyethylene 20 cetyl ether; DPI, diphenylene iodonium; FRE, ferric reductase; FRO, ferric reductase-oxidase; HCF III, ferricyanide; hexacyanoferrate III; ISO, inside-out; ROS, right-side-out; XTT, 2,3-*bis*[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide.

1985; Römheld, 1987). Thus, the function of the standard system appeares not to be linked to iron reduction.

With the establishment of phase-partitioning, the state-of-the-art method for purification of highly enriched plasma membranes (Larsson, 1988), scientists started to investigate electron transport *in vitro*. Data published so far had dealt mainly with *cis*-activities, i.e. donor and acceptor on the same side of sealed membrane vesicles, or total activities in the presence of detergent (Rubinstein and Stern, 1991; Döring and Lüthje, 1996). Only a few attempts have been published that demonstrated a transmembrane electron flow with isolated plasma membranes. Vesicles loaded with an NADH-generating system did not reduce HCF III in the presence of ethanol (Askerlund and Larsson, 1991), while vesicles loaded with NADH by electroporation did, but at a low rate (Böttger *et al.*, 1992). However, a reorientation of vesicles or membrane damage due to the high electric field strength (2 kV cm^{-1}) used for electroporation could not be excluded (Döring and Lüthje, 1996). Plasma membrane vesicles isolated from dicotyledons and loaded with ascorbate during preparation reduced HCF III and other electron acceptors (Hassidim *et al.*, 1987; Askerlund and Larsson, 1991; Asard *et al.*, 1992).

Meanwhile, several redox compounds of plant plasma membranes have been purified and characterized in more detail (Lüthje *et al.*, 1997; Bérczi and Møller, 2000). The predicted iron reductases (FRO1 and FRO2) of strategy I plants are NADPH-dependent flavocytochromes with several transmembrane domains (Robinson *et al.*, 1999; Waters *et al.*, 2001) and homology to yeast ferric reductases, FRE1 and FRE2 (Askwith and Kaplan, 1998).

However, the only redox system with a transmembrane spanning structure that was demonstrated to occur in all plant species and materials so far, was an ascorbate reducible cytochrome *b* (Trost *et al.*, 2000; Bérczi *et al.*, 2001, 2003). From experiments with ascorbate-loaded vesicles, ascorbate was postulated as a major source of electrons in transmembrane redox activity (Asard *et al.*, 1992). On the other hand, ascorbate transporters were identified in plant plasma membranes (Horemans *et al.*, 2000), and there was increasing evidence for an electron transport chain from cytosolic NAD(P)H via a mobile electron carrier to the ascorbate reducible cytochrome *b* (Döring and Lüthje, 1996; Lüthje *et al.*, 1997, 1998; Bérczi and Møller, 2000).

Although *in vivo* data pointed to NAD(P)H as the natural electron donor for the standard system (Qiu *et al.*, 1985; Krüger and Böttger, 1988), a transmembrane electron flow from NAD(P)H to HCF III has not yet been conclusively demonstrated in purified plasma membranes. In the present work, a suitable method was established to investigate transmembrane electron transport *in vitro*. To obtain conditions that allow only transmembrane electron flow, right-side-out (ROS) plasma membrane vesicles isolated from maize (*Zea mays* L.) roots were loaded with NAD(P)H by freeze–thawing. The redox activity of sealed and NADPH-loaded RSO vesicles was modulated by effectors like warfarin [3-(α-acetonyl-benzyl)-4-hydroxycoumarin] or diphenylene iodonium (DPI). The results will be discussed with data published on transmembrane electron transport *in vitro* and with respect to data observed with intact maize roots.

Materials and methods

Plant material and membrane preparation

Maize caryopses (*Zea mays* L. cv. Jet, Saatenunion, Hannover, Germany) were soaked in tap water for about 4 h. The seedlings were germinated at 25 °C in the dark for 5 d. Plasma membranes were purified from roots by aqueous two-phase partitioning as described elsewhere (Lüthje *et al.*, 1998). Purified plasma membranes were stored at –80 °C until use.

ATP-dependent proton transport

ATP-driven proton gradients were measured in the presence of 20 μM acridine orange in HEPES buffer (25 mM HEPES-KOH, pH 7.0, 4 mM $MgSO_4$, 150 mM KCl, 1 mM DTT, and 1 mM EDTA). The reaction was started by the addition of 2 mM ATP and changes in absorbance were detected at 495 nm (Uvikon 720, Kontron, Neuenfahrn, FRG) as described elsewhere (Palmgren *et al.*, 1990). Gramicidin was prepared as a stock solution (2 mg ml^{-1}) in 2-propanol (Sigma, Taufkirchen, FRG).

Redox activity

Transmembrane electron transport was detected with sealed and NAD(P)H-loaded RSO plasma membrane vesicles. Plasma membranes were suspended in phosphate buffer (5 mM phosphate buffer, pH 7.8, 5 mM KCl, and 0.25 M sucrose) and loaded with 25 mM NAD(P)H during four cycles of freezing in liquid nitrogen and thawing in a water bath at 22 °C. Loaded ISO and RSO vesicles were separated by three steps of phase-partitioning on 8 g phase systems (concentrations as described by Lüthje *et al.*, 1998). ISO vesicles of the first lower phase and RSO vesicles of the final upper phase were diluted, collected at 105 000 *g* for 1 h (L-60 ultracentrifuge, Beckman, Munich, FRG), and suspended in phosphate buffer. Before transmembrane measurements, NAD(P)H at the outside of sealed vesicles was oxidized by chasing systems: oxaloacetate and malate dehydrogenase were used for NADH (Böttger *et al.*, 1992), while glucose-6-phosphate dehydrogenase (20 Units, Boeringer, Mannheim, FRG) and 50 μM 6-phosphogluconate (Sigma, Taufkirchen, FRG) were used for NADPH. Equilibration of the reaction was followed at 340 nm with a spectrophotometer (Uvikon 720, Kontron, Neuenfahrn, FRG) under continuous stirring. The custom software (written in Modula 2 by F Hilgendorf, University of Hamburg, FRG) of the computer-controlled photometer allowed switching between two wavelength at time intervals of 10 s during the experiment. NAD(P)H oxidoreductase activity was estimated by the decrease in absorbance for HCF III (ε=1 mM cm^{-1} at 420 nm) and for NAD(P)H (ε=6.22 mM cm^{-1} at 340 nm). Reduction of XTT (2,3-*bis*[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide; ε=21.6 mM cm^{-1}) was measured at 470 nm. Reduction of Fe^{3+}-EDTA (0.2 mM) was measured by the formation of Fe^{2+}-BPDS (ε=25.5 mM cm^{-1}) at 535 nm. Assays were measured in a total volume of 1 ml using half-micro cuvettes (light path, 1 cm).

Rates were calculated and figures were prepared by Microcal Origin™ (Version 5.0, Additive GmbH, Friedrichsdorf/TS, FRG).

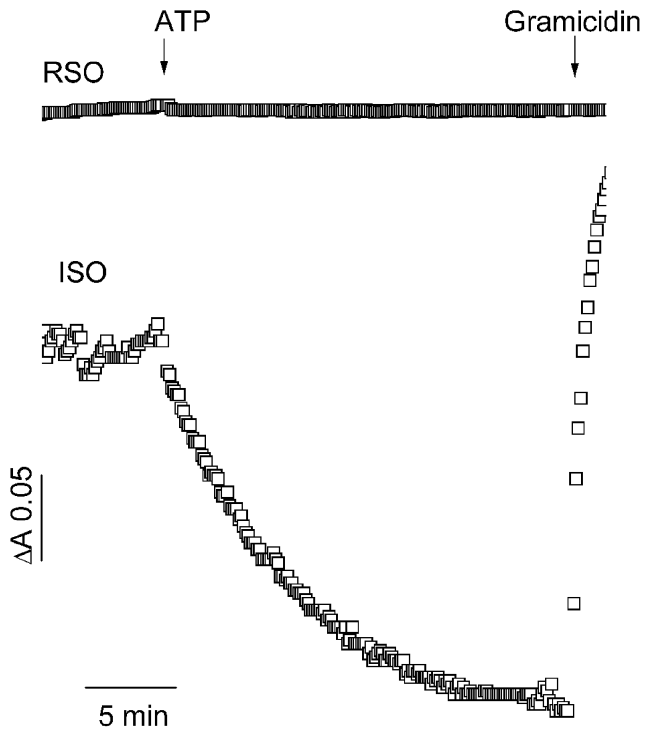

Fig. 1. ATP-driven H^+ gradients of NADPH-loaded plasma membrane vesicles. H^+-pumping activity of plasma membranes was measured with ISO (92 μg protein) and RSO vesicles (130 μg protein). The reaction was started by the addition of 2 mM ATP. Changes in absorbance were detected for acridine orange at 495 nm. The resulting H^+ gradient was destroyed by gramicidin after formation of cation channels.

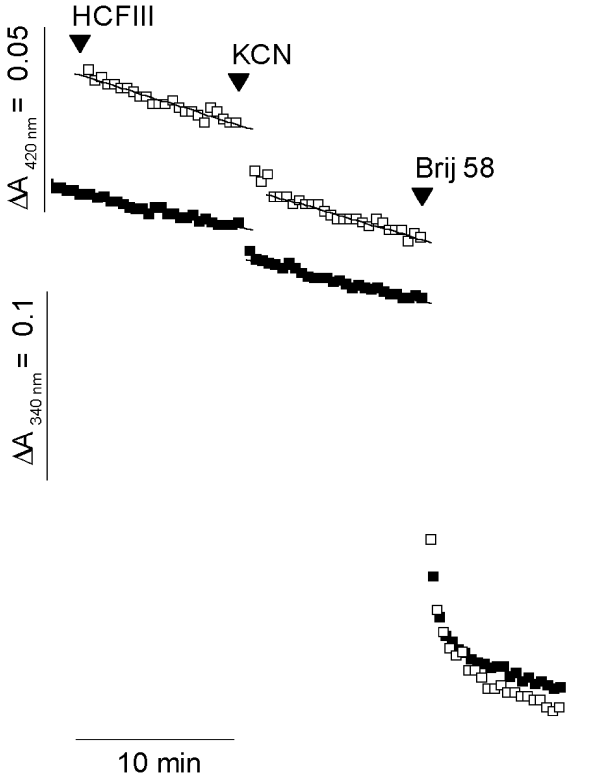

Fig. 2. Transmembrane electron flow in NADH-loaded vesicles. HCF III reductase activity was measured with sealed and NADH-loaded RSO vesicles (140 μg protein). After scavenging NADH outside the vesicles, the reaction was started by the application of 500 μM HCF III and the decrease in absorbance was detected for NADH (340 nm, filled squares) and HCF III (420 nm, open squares) simultaneously. One mM KCN and 0.01% (w/v) Brij 58 were added as indicated by arrows.

Results

After loading with NAD(P)H, plasma membranes were separated into inside-out (ISO) and RSO vesicles by phase-partitioning. In order to prove the status of loaded vesicles, ATP-driven proton gradients were investigated. ISO vesicles produced an ATP-driven proton gradient, while RSO vesicles did not (Fig. 1). Gramicidin completely abolished the proton gradient of loaded ISO vesicles. Comparable results were observed with NADH-loaded vesicles (data not shown).

Before starting transmembrane experiments, excess NAD(P)H on the outside of vesicles was scavenged by chasing systems that do not react with HCF III. A low level of NAD(P)H oxidation remained and, thus a low rate of NAD(P)H oxidation was measurable in the absence of HCF III. After equilibration the reaction was started by the addition of HCF III, which was demonstrated not to penetrate plasma membranes at concentrations used in the present work (Craig and Crane, 1981; Qiu *et al.*, 1985; Giannini and Briskin, 1988; Lüthje and Böttger, 1989). As shown in Fig. 2, transmembrane activity could be detected for several minutes. NADH oxidation was observed simultaneously with HCF III reduction. The ratio of NADH oxidized to HCF III reduced was 0.2. The addition of 1 mM KCN was without significant effect on transmembrane HCF III reduction, either with NADH-loaded (Fig. 2) or with NADPH-loaded vesicles (data not shown). The application of detergent caused a steep decrease in absorbance and both rates were stimulated several-fold. The offset observed for absorbance was due to permeabilization of the membrane. Release of NAD(P)H to the external solution caused a dilution of the substrate, and an additional oxidation by the chasing system and peroxidases located at the apoplastic surface. Furthermore, NAD(P)H dehydrogenases located at the cytoplasmic surface became available and increased the rates of HCF III reduction.

The rates observed with NADPH-loaded RSO vesicles were 10-fold higher compared with those of NADH-loaded vesicles (Table 1). HCF III reduction by NADPH-loaded vesicles increased with higher K^+ concentrations, rates of 102.4 ± 11.5 nmol min^{-1} mg^{-1} protein (n=3) and 126.3 ± 6.8 nmol min^{-1} mg^{-1} protein (n=3) were detected at 5 mM and 150 mM KCl, respectively. Fe^{3+}-EDTA was not reduced at significant rates with NADPH-loaded vesicles (data not shown), whereas XTT was reduced at 765 ± 77 pmol min^{-1} mg^{-1} protein (n=4).

Table 1. *Transmembrane electron transport in dependence on the donor*

HCF III reduction by maize roots was measured with sealed and NAD(P)H-loaded RSO vesicles in phosphate buffer (5 mM phosphate, pH 7.8, 5 mM KCl, and 250 mM sucrose). The reaction was started by the application of 500 µM HCF III. Data presented are the mean ±SD of *n* experiments measured with two independent membrane preparations; ADH, alcohol dehydrogenase.

Species	Tissue	Donor	Concentration [mM]	HCF III reduction (nmol min^{-1} mg^{-1} protein)	References
Gossypium hirsutum L.	Root	Ascorbate	100	0.021	Hassidim *et al*., 1987
Phaseolus vulgaris L.	Hook	Ascorbate	100	9.2	Asard *et al*., 1992
Beta vulgaris L.	Leaf	NAD^+ +ADH	10	n.d.	Askerlund and Larsson, 1991
Glycine max L.	Hypocotyl	NADH	20	2.9±2.1 (*n*=3)[e]	Böttger *et al*., 1992, 1995
Zea mays L.	Root	NADH	25	7.9±2.4 (*n*=2)	
		NADPH	25	102.4±11.5 (*n*=3)	

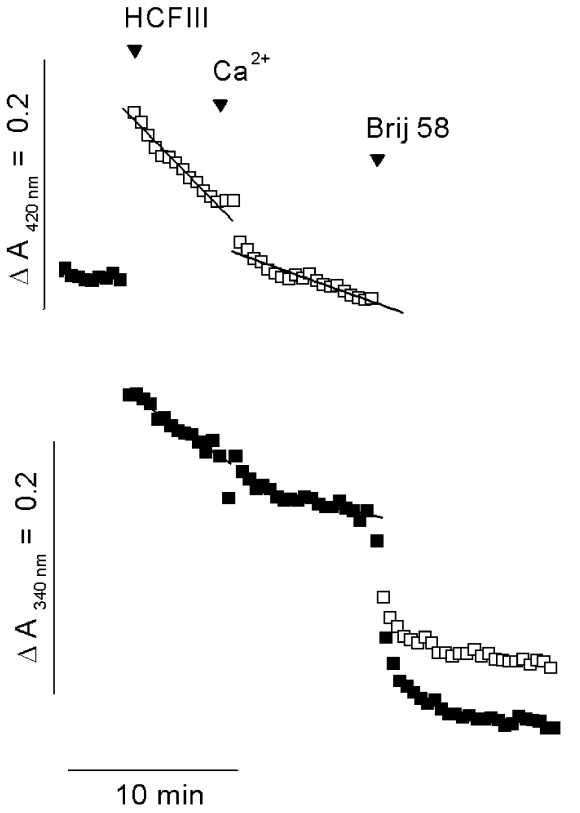

Fig. 3. Transmembrane electron flow in NADPH-loaded vesicles. HCF III reductase activity was measured with sealed and NADPH-loaded RSO vesicles (140 µg protein). After scavenging NADPH outside the vesicles, the reaction was started by the application of 500 µM HCF III. The decrease in absorbance was simultaneously detected for NADPH (340 nm, filled squares) and HCF III (420 nm, open squares). 100 µM calcium and 0.01% (w/v) Brij 58 were added as indicated by arrows. Effects were reproducible with independent membrane preparations.

As shown in Fig. 3, calcium inhibited the HCF III reduction with NADPH-loaded vesicles after a short *lag*-phase of about 2 min. The ratio of NADPH to HCF III was 0.16 before and 0.32 after the application of calcium. The addition of Brij 58 (polyoxyethylene 20 cetyl ether) caused a steep decrease in absorbance for both NADPH and

Table 2. *Modulation of transmembrane HCF III reductase activity*

After chasing, NADPH-loaded RSO vesicles were preincubated for 3 min with the effectors. The reaction was started by the addition of 500 µM HCF III and reduction was detected at 420 nm in phosphate buffer (5 mM phosphate, pH 7.8, 150 mM KCl). Data presented are the mean ±SD of *n* experiments. Effects were reproducible with two independent membrane preparations.

Effector	Concentration	HCF III reduction (nmol min^{-1} mg^{-1} protein)
Control	–	126.3±6.8 (*n*=3)
Calcium	100 µM	43.0±13.6 (*n*=2)
DPI	200 µM	94.9±26.8 (*n*=2)
Warfarin	200 µM	88.7±9.1 (*n*=2)

HCF III. Calcium inhibited transmembrane HCF III reductase activity to 68% with NADPH-loaded vesicles (Table 2) and by about 35% in NADH-loaded vesicles. A weak inhibition of NADPH-driven transmembrane electron flow was observed after preincubation with warfarin (34%) or DPI (25%).

Discussion

In the present work, a significant transmembrane electron flow in NADPH-loaded plasma membrane vesicles, with rates of HCF III reduction comparable to the rates observed *in vivo*, is demonstrated for the first time. The results for ATP-driven proton gradients (Fig. 1) and the detergent effect observed for transmembrane experiments demonstrated that the membrane vesicles used in the present study were sealed and mainly RSO oriented (Figs 2, 3). Any excess of NAD(P)H outside the sealed vesicles after loading was probably removed during the separation of ISO and RSO vesicles by phase-partitioning. The additional use of appropriate chasing systems should have removed any leakage of NAD(P)H instantaneously.

The low rate of NAD(P)H oxidation observed in the absence of HCF III may be due to NAD(P)H oxidase

activities present in plasma membranes (Lüthje *et al.*, 1997; Bérczi and Møller, 2000). This hypothesis also explained the observed ratios of NAD(P)H to HCF III in the present work. The NADPH to HCF III stoichiometry of 0.16 reported here is well below the value expected for a one electron transfer (0.5). The difference is most probably due to a systematic underestimation of NADPH trapped inside the tiny vesicles. With vesicle sizes being in the same order of magnitue as the wavelength of light, significant scatter and interference terms will be introduced into the photometric system. In fact, such effects have been predicted before (Böttger, 1989). The reduced sensitivity of photometry for substances inside vesicles is the physical basis for the determination of proton gradients by measuring Acridine orange accumulation (Palmgren, 1991). Ratios observed with NADPH-loaded vesicles increased 2-fold after the addition of calcium, indicating additional consumption of NADPH. Since the concentration of NAD(P)H inside the vesicles was in the millimolar range, plasma membrane-bound peroxidases could have consumed part of it. However, peroxidases were suggested to be located at the apoplastic surface of plasma membranes and should be inhibited by cyanide (Vianello and Macri, 1991; Mika and Lüthje, 2003). Since the application of the peroxidase inhibitor cyanide was without an effect (Fig. 2), chasing systems seemed to work properly and NAD(P)H was apparently not available at the outer surface of NAD(P)H-loaded RSO vesicles. On the other hand, cyanide-insensitive NAD(P)H oxidase activities have been described for plant plasma membranes (Van Gestelen *et al.*, 1997; Bolwell *et al.*, 1998) and may well have accounted for the observed NAD(P)H consumption.

Initial attempts to establish a transmembrane electron transport *in vitro* have been published (Hassidim *et al.*, 1987; Askerlund and Larsson, 1991; Asard *et al.*, 1992; Böttger *et al.*, 1992, 1995). Table 1 lists the rates observed for HCF III reduction with vesicles loaded by different methods compared with data observed in the present study. Rates of ascorbate-loaded and NADH-loaded RSO vesicles were comparable, while the HCF III reduction of NADPH-loaded vesicles was several-fold higher. Due to this fact it seemed likely that NADPH had a major function in donating electrons for transmembrane redox activity. This hypothesis was supported by *in vivo* data where the internal NAD(P)H level decreased after the application of HCF III (Qiu *et al.*, 1985; Krüger and Böttger, 1988).

As mentioned above, potassium was needed to observe a sufficient transmembrane reaction. This observation confirmed data published for ascorbate-loaded vesicles (Table 1). The sodium salt of HCF III was reduced at very low rates (Hassidim *et al.*, 1987), while reduction of the potassium salt occurred at 10-fold higher rates (Asard *et al.*, 1992). Furthermore, the reduction of 2,6-dichlorophenolindophenol-3′-sulphonate by ascorbate-loaded vesicles was stimulated several-fold by the K^+ ionophore valinomycin, which was included to abolish a membrane potential (Askerlund and Larsson, 1991). These data supported the hypothesis of an electrogenic transmembrane electron flow in NAD(P)H- or ascorbate-loaded RSO vesicles to HCF III. Furthermore, osmotic conditions were important for the detection of NAD(P)H inside loaded vesicles, because shrinking (<340 nm) or swelling (i.e. light-scattering) interfere with the sensitivity of NAD(P)H detection.

Bivalent cations stimulated transmembrane HCF III reduction of intact plants at concentrations above 0.1 mM, but no supraoptimal concentration was seen up to 40 mM calcium (Rubinstein *et al.*, 1984; Böttger and Hilgendorf, 1988). This result was interpreted as an unspecific effect of cations by screening the negative surface charge of the plasma membrane. On the other hand, transmembrane electron transport in NADPH-loaded vesicles was significantly inhibited by 100 μM calcium (Table 2). The *lag*-phase shown in Fig. 3, suggested a binding-site for calcium at the cytoplasmic surface of the membrane. However, NAD(P)H oxidation was less affected compared with HCF III reduction. Thus the NADPH to HCF III ratio increased 2-fold. This observation indicated additional electron transfer to an alternative electron acceptor, i.e. oxygen. Calcium binding by proteins may have caused conformational changes and, thereby, a stimulation or inhibition of activity. The occurrence of a calcium-dependent protein kinase was demonstrated in oat plasma membranes (Schaller *et al.*, 1990) and a direct regulation of a respiratory burst oxidase homologue (Rboh) in plant plasma membranes by calcium was suggested to be important for the rapid stimulation of the oxidative burst during hypersensitive response in plants (Lamb and Dixon, 1997). However, the rates observed with XTT as an indicator of superoxide production were several-fold lower compared to the rates of the phagocyte NADPH oxidase, which might suggest a function in signalling rather than an oxidative burst.

Coumarins have been described as inhibitors of HCF III reductase activity for intact maize roots (Döring and Lüthje, 1996). As shown in Table 2, pre-incubation with warfarin significantly decreased the rate of HCF III reduction by NADPH-loaded vesicles. The effect was comparable with the inhibition observed with intact maize roots (Döring *et al.*, 1992). It is not clear which redox component(s) contributed to transmembrane activity, but a coumarin-sensitive NADPH oxidoreductase has not been identified in plant plasma membranes so far. The effect of DPI on transmembrane HCF III reduction with NADPH-loaded vesicles was weak (Table 2). The inhibition occurred in the presence of reduced flavins indicating that flavoproteins may have partially contributed to transmembrane HCF III reductase activity. A DPI-sensitive NAD(P)H:quinone oxidoreductase was identified in

plasma membranes isolated from zucchini (*Cucurbita pepo* L.) hypocotyls (Trost *et al.*, 1997). The partially purified protein was active with hydrophilic benzoquinones. However, benzoquinones were not present in the plant plasma membrane and a possible function of the natural occurring vitamin K (Lüthje *et al.*, 1998) as a substrate of plasma membrane-bound redox proteins and in transmembrane electron flow has not yet been demonstrated.

Vesicles isolated from iron-sufficient dicotyledons and loaded with an NADH-generating system did not reduce Fe^{3+}-citrate in the presence of ethanol, while rates of 1–2 nmol Fe^{2+}-BPDS min^{-1} mg^{-1} protein were detected with ascorbate-loaded vesicles (Hassidim *et al.*, 1987; Askerlund and Larsson, 1991). NADPH, however, has never been tested for transmembrane iron reductase activity in strategy I plants *in vitro*. The negative result for Fe^{3+}-EDTA reduction with NADPH-loaded vesicles isolated from maize roots might be due to the different iron uptake strategy evolved by grasses. The data confirm results observed with intact maize roots (Bienfait, 1985).

NADH-dependent HCF III reduction was demonstrated by electroporation experiments, but the rates were lower compared with the rates observed in the present work (Table 1). The contradicting results observed for HCF III reduction with NADH-loaded vesicles versus the NADH-generating system (Table 1) indicated possible side-effects of alcohol on transmembrane electron flow (Askerlund and Larsson, 1991; Döring and Lüthje, 1996).

In conclusion, NAD(P)H-loaded RSO vesicles prepared by freeze–thawing are a suitable system to study transmembrane electron flow *in vitro*. Rates of HCF III reduction were several-fold higher with NADPH-loaded vesicles compared with other electron donors, i.e. NADPH seemed to have a major function in transmembrane electron transport activity, at least in maize roots. Since NAD(P)H oxidation also occurred in the absence of artificial electron acceptors and ratios of NAD(P)H to HCF III did not match with the theoretical values, oxygen seemed to be a possible substrate of the standard system. The effects of inhibitors observed with NADPH-loaded vesicles were consistent with *in vivo* experiments. However, the structure, natural acceptor, and physiological function(s) of the standard system need further investigation. The method established in the present work will be a useful tool for those experiments.

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