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The effect of metabolic inhibitors, sugars and fusicoccin on the electrical potential difference arising across an intact *Chenopodium rubrum* L. plant

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Abstract

An analysis of the effect of metabolic inhibitors, sugars, and fusicoccin on the trans-plant electrical potential difference arising across one-week-old green or herbicide-treated *Chenopodium rubrum* L. plants was performed. The substances were applied either to the solution bathing the root or in the form of drops to the stem. The respiratory inhibitors (KCN and salicylhydroxamic acid), sulfhydryl agents (N-ethylmaleimide and *p*-chloromercuribenzene sulfonic acid) and proton ionophore (carbonyl cyanide *m*-chlorophenylhydrazone) affected the electrical potential, the kinetics of the induced changes varying with different inhibitors and site of application. None of the applied sugars (sucrose, glucose or sorbitol), ATPase stimulator fusicoccin or inhibitor vanadate exerted any appreciable effect on the electrical potential. An effect of sucrose could be observed in the case of its application immediately following de-rooting, especially in the case of herbicide-treated plants. These results we explain by non-participation of the sucrose transporter or the proton ATPase in the generation of the electrical potential difference across intact plants (apoplast-apoplast configuration).

Additional key words: apoplast, ion transport, respiration, symplast.

Introduction

A number of electrophysiological measurements on intact higher plants was performed using extracellular apoplastic electrodes (Jaffe 1968, Greppin *et al.* 1973, Giroldini 1988, Adamec and Krekule 1989b, Stanković *et al.* 1997). Intracellular recordings (Novak and Greppin 1979, Montavon *et al.* 1983, Adamec and Krekule 1989a), performed simultaneously with extracellular ones (Zawadzki and Trebacz 1985, Ullrich and Novacky 1990, Frachisse-Stoiljsković and Julien 1993, Polevoi *et al.* 1996), demonstrated the existence of a strong correlation between the transients in the extracellular and the transmembrane electrical potential difference. Surface potential measurements sometimes can be of advantage over intracellular ones, because they are non-invasive and can provide relatively long lasting measurements on a large number of cells (Due 1993).

In our previous publications, we have analysed the light-induced transients of the trans-plant electrical potential difference occurring in the intact *Chenopodium rubrum* L. plants, linking them to the photosynthetically-induced ionic movements in the cotyledons (Živanović *et al.* 1992). Also, we have associated the spontaneously generated transients, occurring predominantly in the light, with the induction of flowering (Živanović and Vučinić 1996). The aim of the present study was to further characterise the mechanisms of generation of the electrical potential arising across intact *Chenopodium* plants, by application of various substances known to affect the membrane-associated electrical potential generators of the plant cells.

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Abbreviations: APW - artificial pond water; CCCP - carbonyl cyanide *m*-chlorophenylhydrazone; MES - 2-(N-morpholino) ethanesulfonic acid; NEM - N-ethyl maleimide; PCMBS - *p*-chloro-mercuribenzene sulfonic acid; SHAM - salicylhydroxamic acid; TPP - trans-plant electrical potential difference; Tris - tris-(hydroxymethyl) aminomethane.

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Materials and methods

The experiments were performed on intact plants (except where otherwise specified) of *Chenopodium rubrum* L., selection 184. Three days after sowing, the plants were transferred to a growth chamber (temperature 20 °C, relative humidity 70 %, irradiance 10 W m^{-2}) on a nylon mesh ring filled with 0.6 % agar and floating on Hoagland's nutrient solution under continuous light, as previously described (Živanović and Vučinić 1996). Six-day-old plants were mounted, together with their agar-filled ring carrier, into the experimental chamber containing initially artificial pond water (APW) as the bathing solution (1 mM $CaCl_2$, 1 mM KCl). The pH of APW was adjusted with MES/Tris buffers (2 mM) to pH 5.5 or 7.0. Plants incubated in herbicide SAN 9789 (0.01 mM) did not synthetise chlorophyll. Electrical potential difference across the plant (TPP) was measured by extracellular Ag/AgCl macroelectrodes that were in contact with the bathing solution and cotyledon tip via liquid filled chambers. The experiments were performed in a light-proof Faraday cage in which the temperature was kept constant at 25 °C, according to the method previously described (Živanović *et al.* 1992). The plants in experimental chambers were exposed to dark/light/dark cycles before the application of chemicals in order to test the contact of the plant with the solution. The data presented were obtained in darkness, except where explicitly stated. The irradiance (provided by a *Schott KL150* illuminator via a light guide) was 10 W m^{-2} at the cotyledon surface. The effect of various substances tested on the TPP was analysed by their addition to the stirred solution bathing the roots, or in the form of a drop (0.025 cm^3) placed at the stem of the plant, into a small well (0.050 cm^3 volume) in the agar/nylon mesh (Fig. 1). Chemicals were added as soon as the steady-state TPP was obtained under continuous darkness. Following the attainment of a steady state, respiratory inhibitors (20 mM KCN, 20 mM SHAM), proton ionophore (0.01mM CCCP), ATPase inhibitor (1 mM vanadate) and stimulator (0.01mM fusicoccin), sugars (100 mM sucrose, 100 mM glucose or 100 mM sorbitol) or sulfhydryl agents (1 mM NEM, 1mM PCMBS) were applied to the root or to the stem. In the experiments with de-rooted plants, the roots protruding from the agar mesh into the bathing solution were excised 2 mm below the bathing solution surface, and the effect of addition of substances analysed after a steady-state value of TPP was attained (in approximately 2 h after switching off the light). TPP recordings were digitized, and the traces of 5 - 6 experiments were averaged using *Statgraphics* program (*Statistical Graphics Corporation*, Rockville, USA).

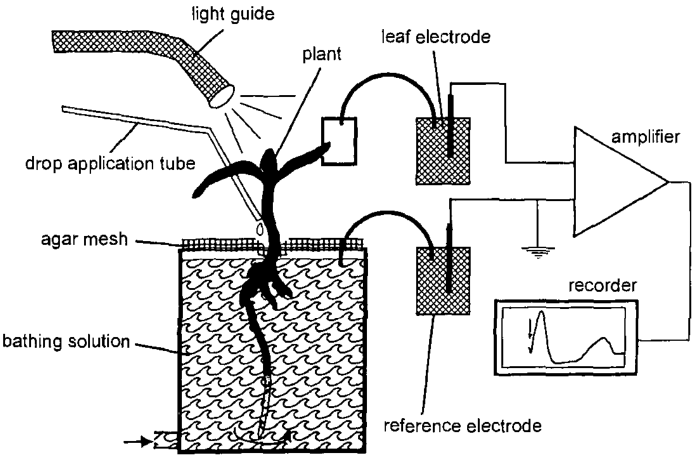

Fig. 1. Schematic representation of the experimental setup used for trans-plant electrical potential difference measurements.

Results

The potential difference gradually reached the resting state after the light to dark transition in about 120 min. The steady-state level of the potential difference under continuous darkness was similar at two tested pH values, being -36.9 ± 1.8 mV (*n* = 87) at pH 7 and -36.6 ± 1.3 mV (*n* = 119) at pH 5.5; the electrode in contact with the root being taken as the reference electrode.

The effect of the inhibitors, tested at two pH values (5.5 and 7.0), was not pH dependent, except the effect of SHAM which was more pronounced at pH 5.5 as compared to pH 7.0 (Fig. 2). Since no significant differences in the effect of the other substances at pH 5.5 and pH 7.0 could be observed, only the data at pH 5.5 are shown for clarity. The inhibitors induced a depolarisation of the electrical potential varying in magnitude, with the exception of vanadate and PCMBS, applied to the root, inducing a slight hyperpolarisation (up to 10 mV). Generally, the sulfhydryl reagents (membrane penetrating NEM, or non-penetrating PCMBS), as well as the ATPase inhibitor, vanadate, applied either to the root or to the stem induced very gradual potential changes of low magnitude (10 - 18 mV). The ATPase stimulator fusicoccin did not exhibit any appreciable effect. Treatment of the roots with 0.01 mM CCCP induced a gradual depolarisation of TPP up to 18 mV. CCCP addition to the stem induced a transient whose kinetics was different from that obtained in the case of the root application, with a rapid initial phase being observed in the first 5 min, followed by a more gradual depolarisation reaching a maximum of 22 mV in about 20 min, and subsequent slow repolarisation.

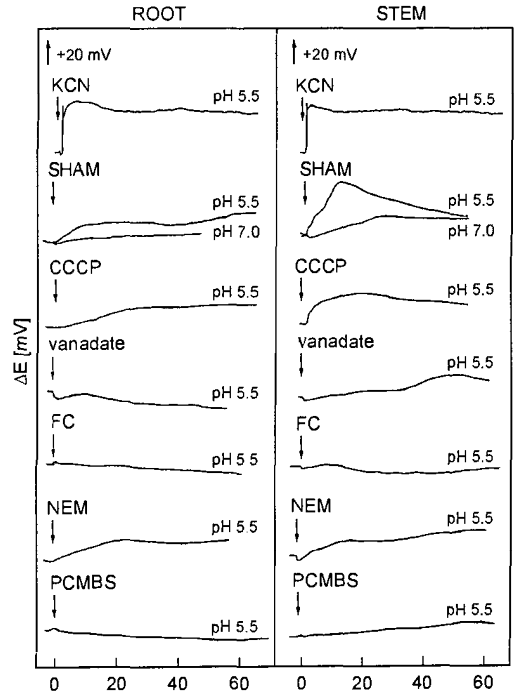

Fig. 2. Effects of metabolic inhibitors (20 mM KCN, 20 mM SHAM, 0.01 mM CCCP, 1 mM vanadate, 1 mM NEM, 1 mM PCMBS) and fusicoccin (0.01 mM) on the trans-plant electrical potential difference of intact *Chenopodium* plants. The substances were added to the solution (1 mM $CaCl_2$, 1 mM KCl) bathing the roots or to the stem in the form of a drop. ΔE - difference in trans-plant potential from the steady-state values obtained before the addition of inhibitors. The *vertical arrows* indicate the moment of substance addition to the bathing solution or to the stem.

The addition of respiratory inhibitor KCN (20 mM), either to the stem or to the root, resulted in a strong (about 40 mV) and rapid (1 - 5 min) depolarisation of TPP followed by a slight repolarisation. Lower concentrations of KCN (1 - 10 mM) did not produce such considerable effects on TPP (data not shown). Control experiments with KCl up to the concentrations of 100 mM did not show any appreciable effect on TPP (data not presented). The effect of the second respiratory inhibitor, 20 mM SHAM, demonstrated a considerable dependence on the pH and site of application. Initial depolarisation induced by SHAM was the most pronounced at pH 5.5 applied to the stem, with maximum induced depolarisation of about 40 mV in 12 - 15 min. In that case, the depolarisation was followed by pronounced repolarisation. Herbicide-treated plants showed a different effect of SHAM at root application as compared to the green plants, while KCN exerted a similar effect in both cases (Fig. 3).

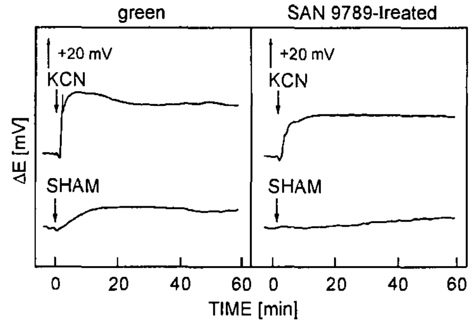

Fig. 3. The effect of respiratory inhibitors KCN and SHAM (20 mM), added to the solution bathing the roots, at pH 5.5 on the trans-plant electrical potential difference of green and herbicide-treated intact plants of *Chenopodium*. For the other see Fig. 2.

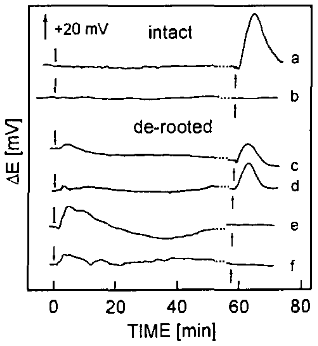

Fig. 4. The effect of 100 mM sucrose, added to the solution bathing the roots, on the trans-plant electrical potential difference of intact and de-rooted *Chenopodium* plants. The plants were de-rooted 2.5 h (*c,e*) or 5 h (*d,f*) before sucrose addition. Either green (*a,c,d*) or herbicide-treated (*b,e,f*) plants were used. Downward and upward pointing *arrows* indicate the moment of sucrose addition and the beginning of illumination, respectively. For the other see Fig. 2.

Contrary to metabolic inhibitors, sugars applied either to the root (Fig. 4*a*) or to the stem (data not presented), exerted no effect on TPP of intact green plants. We applied sucrose, glucose and sorbitol (to test the possible osmotic effect) at concentrations up to 1 M, and in no case could any effect of sugars be observed (data not presented). Intact herbicide-treated plants did not show any effect of sugars either (Fig. 4*b*). The light-induced electrical transient had the typical pattern in the case of green plants and was absent in the case of herbicide-treated plants (Živanović *et al.* 1992). In the case of de-rooted plants, sucrose addition to the bathing medium produced a response of TPP, typical for a sucrose response (Fig. 4*c*). This effect was even more pronounced in the case of herbicide-treated plants (Fig. 4*e*). As the plants regenerated their wounded tissue, and presumably closed the symplastic pathway to the environment, the sucrose-induced response gradually disappeared (Fig. 4*d,f*). Excision of roots in green plants did not affect light-induced transients (Fig. 4*c,d*).

Discussion

In the early seventies there have been a number of attempts to utilize macroelectrode measurements of the trans-plant potentials in the studies of the light-induced transients. Greppin *et al.* (1973) linked such transients with the photoperiodic induction of plants. Our results on *Chenopodium* (Živanović *et al.* 1992) have demonstrated the participation of the photosynthesis in the light-induced transients, but no participation of phytochrome in the generation of the trans-plant electrical potential difference could be observed. In this article we performed a further characterisation of the TPP utilizing metabolically active substances.

High sensitivity of TPP to respiratory inhibitor KCN argues in favour of the involvement of an electrogenic system in the generation of this TPP. The magnitude of depolarisation induced by 20 mM KCN in our experiments was of similar kinetics to the depolarisation of pea root membrane potential induced by 1 mM KCN. The immediate effect of KCN could be ascribed to a direct interaction with some plasma membrane electrogenic mechanism, rather than an indirect effect of cyanide on the metabolic processes supplying energy to the ion uptake systems (Anderson *et al.* 1974). The relatively low sensitivity of TPP to the inhibitor of plasma membrane ATPase indicates that the ATPase does not play a dominant role in the generation of TPP. The effect of 1 mM vanadate on TPP was comparable to depolarisation of transmembrane potential of pea cortical cells in germinated seeds and cortical cells of maize roots induced by 0.5 mM vanadate that inhibited membrane ATPase activity (Cocucci *et al.* 1980). Also, the slight effect of sulfhydryl reagents, known to inactivate the ATPase (Beffagna *et al.* 1979, Katz and Sussman 1987) and decrease the ATP level (Lichtner *et al.* 1981), suggests the involvement of some electrogenic system(s) which are not dependent on the ATPase activity. Similar effects of the inhibitors (KCN, vanadate, sulfhydryl) on the TPP were observed when applied to the cut end of excised maize root, demonstrating the participation of an electrogenic system other than the ATPase in the generation of longitudinal component of trans-root potential difference (Vuletić and Vučinić 1997/98). The initial depolarisation of TPP induced by CCCP applied to the stem (about 22 mV), although much lower than the depolarisation of transmembrane potential in *Riccia* thallus cells (about 100 mV), is probably caused by the disruption of the proton gradient across the plasma membranes (Felle and Bentrup 1977). This result, as well as the low effect of ATPase inhibitors, implicate some additional proton pumping system in the generation of the TPP. In general, the inhibitors used induced similar changes in TPP when compared to intracellular measurements performed by other authors, although the concentrations of the inhibitors used were usually higher (*e.g.* KCN).

A different effect was observed when SHAM was applied to the root and stem of green plants (Fig. 2). Also, a difference between green and herbicide-treated plants could be seen (Fig. 3). These differences can be explained by two mechanisms. SHAM is a well-known inhibitor of the alternative respiratory pathway (Lambers *et al.* 1983), and our results could indicate the participation of different respiratory mechanisms in the root and stem. A similar explanation could be applied to herbicide-treated plants as an implication of the engagement of different respiratory pathways in the green (autotrophic) and herbicide-treated (heterotrophic) plants. However, SHAM can also interfere with NAD(P)H-dependent peroxidase activity (Vianello and Macri 1991). This peroxidase is active at acidic pH (< 6 pH, Hadži-Tašković Šukalović and Vuletić 1998). The significant depolarisation at pH 5.5 obtained in our experiments (much more pronounced than that observed at pH 7) when SHAM was applied to the stem, might be an indication of involvement of stem peroxidase in the generation of TPP, similarly to the case of the trans-root potential (Vuletić and Vučinić 1996, 1997/98). Further experiments are needed to resolve this dilemma.

Contrary to the metabolic inhibitors, sucrose applied to the root or to the stem did not exhibit any effect on the TPP of intact plants. Sucrose transport across cell membranes, that occurs by co-transport with H^+ ions, is a well-documented (Lalonde *et al.* 1999) and transient depolarisation of membrane potential (Slayman and Slayman 1974, Novacky *et al.* 1978, Felle and Bentrup 1980), as well as the trans-organ electrical potential differences across roots (Vučinić and Vuletić 1995) or cotyledons (Vreugdenhil and Spanswick 1988) were observed. It is hard to believe that such transport is lacking in *Chenopodium* plasma membranes. Also, fusicoccin has been shown to activate the proton ATPase of plants (Beffagna *et al.* 1977) and affects the membrane electrical potentials (Cocucci *et al.* 1976). The lack of effect of these substances on the TPP, indicates that the charge transfer, which accompanies the sucrose transport, is present in the form of a local internal electrical current, not influencing the net transfer of current along the intact plant.

We have previously shown that the light-induced transients in TPP were photosynthetic by origin, and that the herbicide-treated plants do not exhibit such transients (Živanović *et al.* 1992). Shabala and Newman (1999) have resolved such transients, using combined micro- and vibrating ion-selective electrodes on partly extracted epidermal and mesophyll cell layers, into their components and linked them with fluxes of protons, calcium, potassium and chloride. In the present experiments (Fig. 4) we show that de-rooting of the plants does not affect this response, demonstrating that the light-on-off responses are independent of the intactness of the plant.

Thus, our results with de-rooted plants and the lack of sucrose or fusicoccin effect on the TPP argue in favour of a concept implying that the trans-organ electrical potentials are a symplast-apoplast phenomenon, not observable in case of electrophysiological apoplast-apoplast measurements. The differential effect of metabolic inhibitors applied to the stem or the root indicates a heterogeneous coupling of the translocation and ion-uptake components of TPP to the metabolic processes within the cell.

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