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Effect of ouabain on stomatal movements and transpiration rate of *Secale cereale*

A. MORANT-AVICE*, P. JURVILLIERS*, G. TREMBLIN* and A. COUDRET**

*Laboratoire de Physiologie Végétale, Université du Maine, Avenue Olivier Messiaen, BP 535, 72017 Le Mans Cedex, France**
*Laboratoire de Physiologie et Biotechnologie Végétales, Université de Clermont Ferrand, 4 Rue Ledru, 63038 Clermont Ferrand, France***

Abstract

Young rye seedlings (*Secale cereale* cv. Petkus) were grown for 12 or 18 d in aerated nutrient solution. CO_2-free air in light or in darkness induced stomatal opening. When light and CO_2-free air were applied together, maximum stomatal opening was observed. Na^+ channel inhibitor [^{3}H]ouabain was taken up from nutrient medium and translocated into the leaves *via* the xylem sap. The presence of 10^{-5} M ouabain in the root medium for 20 h increased stomatal opening and transpiration rate. On the other hand, stomatal closing was not affected by the presence of ouabain. These results suggested that Na^+ might play a role in stomatal movements.

Additional key words: Na^+ flux, rye.

Introduction

Uptake of ions and production of osmotically active organic compounds (for review see, *e.g.*, Mansfield *et al.* 1990) drive water influx, resulting in guard cell swelling and stomatal opening. During stomatal opening, membrane hyperpolarization by an H^+-ATPase (Assman *et al.* 1985) creates an electrical gradient for K^+ uptake *via* K^+ channels. Schwartz *et al.* (1995) suggested that anion channels can function as negative regulator of stomatal opening, prohibiting ion uptake into guard cells.

Willmer and Mansfield (1969) have shown that Na^+ was more effective than K^+ in stomatal opening of isolated epidermis of *Commelina communis*. However, Garrec *et al.* (1983) have not observed a significant change in Na^+ content between open and closed stomata of *Vicia faba* and *Commelina communis*. In an attempt to show the role of Na^+ flux in stomatal movements and transpiration rate of a whole plant

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*Fax (+33)43 83 32 45.

we have applied ouabain (Na^+ channel inhibitor of animal cells, Hammond and Tritsch 1990) in root nutrient solution of *Secale cereale* cv. Petkus, able to grow in a salt medium (Morant-Avice *et al.* 1989). The treatment of plants with ouabain resulted in reduction of the translocation of saccharides in sunflower (Shiroya and Kura 1979), affection of the membrane potential at low and high pH in *Nitellopsis obtusa* (Stolarek 1977), and inhibition of active efflux of Na^+ in plasmalemma of root cells of carrot, but not K^+ influx (Cram 1968). On the other hand, Tikhaya *et al.* (1976) demonstrated the existence of ouabain sensitive Na^+/K^+ ATPase activity in cell membranes isolated from barley roots.

Materials and methods

Plants and growing conditions: Seeds of *Secale cereale* L. cv. Petkus (2n = 14, genomes RR) were supplied by the Groupe d'étude et de contrôle des variétés et des semences, Guyancourt, France. They were germinated in Petri dishes in the presence of distilled water before transfer to hydroponic culture on Coic and Lesaint (1973) medium in a growth chamber. The photoperiod was 16 h; irradiance at the collar level was 280 μmol(PAR) m^{-2} s^{-1} (400 W *Phytoclaude* lamps), temperature and relative humidity of air were 22 °C and 33 % during the light period and 18 °C and 50 % during the night period.

Transport of [^{3}H]ouabain: For testing and quantifying ouabain transport from culture solution to the leaves of young rye, 18 d-old plants were transferred to a nutrient medium containing [^{3}H]ouabain (3.5 kBq cm^{-3}, specific activity 351.2 GBq $mmol^{-1}$) titrated to the appropriate concentration (10^{-5} M) with unlabelled ouabain and treated for 1, 4, 10, 16, 24 or 28 h in the conditions of the growth chamber. Then, leaves were collected, quickly weighed (fresh mass), cut off, dipped into liquid nitrogen and powdered in a mortar; the [^{3}H]ouabain was extracted with 50 % ethanol and 1 % toluene (v/v) in water solution.

The filtrate obtained (1 cm^3) was placed in a scintillation vial with 10 cm^3 of hydrophilic liquid scintillation cocktail (*Hydratron*, Kontron, France) radioactivity was measured (*Betamatic I*, Kontron, France) and quench corrected; quench curves were established with (unlabelled) coloured leaf extracts using external standard ratio method. It was checked that the [^{3}H]ouabain in the insoluble fraction was negligible. All assays were run in triplicate. The results are given in radioactivity [kBq] incorporated in 1 g of fresh mass of leaves harvested.

Autoradiographs: Each radioactive leaf (detached from 28 h treated plants) was exposed to hyperfilm [^{3}H] (*Amersham*) for about 60 - 80 d at -80 °C. The leaves were traced off on tracing paper prior to autoradiography to permit comparison of the leaf morphology with the location of radioactivity.

Stomatal permeability and transpiration rate: Stomatal permeability was measured on 18-d-old plants with 6 - 7 leaves with a hydrogen porometer (Louguet 1969). The coefficient of permeability [cm] is the foliar permeability [cm^3 s^{-1}] divided by the

diffusion coefficient for H_2 in air [cm^2 s^{-1}]. The permeability coefficient is proportional to the average opening of stomata.

The experimental chamber (Lascève and Couchat 1980) divided into two independent compartments for shoot and roots allowed continuous measurement of transpiration of the whole plant (12-d-old, 3 - 4 leaves) with a dew point hygrometer (*General Eastern*, Elcowa, France).

Experimental procedure: Experiments began at 09.00, after 8 h of darkness in ambient air. Ouabain (10^{-5} M) was added to the nutrient solution 20 h before the application of the stomatal opening treatments. Each experiment was repeated at least 3 times with different plants.

Results

Ouabain incorporation and movement in the plant: During the first hours of illumination, the radioactivity fixed in the leaves slightly increased with time (Fig. 1). The activity [5.5 kBq g^{-1}(f.m.)] measured at the end of the photoperiod did not significantly increase during the dark. Then, after lighting, the movement of [^{3}H]ouabain to the leaves seemed to start again. These results showed that ouabain from culture medium was transported to the leaves by the root system. A principal location of radioactivity was confirmed by autoradiography along the vascular bundles (result not shown). Thus, to study the effect of ouabain on stomatal opening and transpiration rate, ouabain was introduced into the root medium 20 h before the beginning of the experiments.

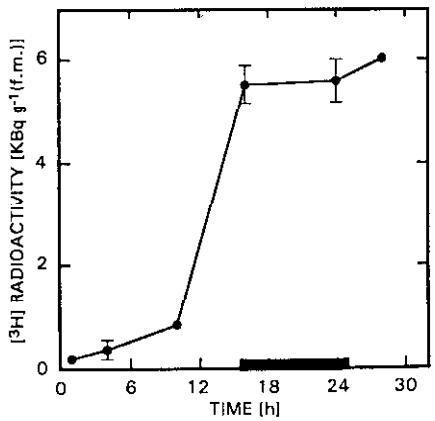

Fig. 1. Time-course of [^{3}H]ouabain accumulation into leaves of 18-d-old rye. The *black line* on the abscissa indicates the period of darkness. *Vertical bars* − ± S.E.

Maximum stomatal aperture was obtained in light with dry CO_2-free air (Fig. 2*A*). The presence of ouabain further enhanced stomatal opening (+20 %) The maximum aperture was reached within 30 min in plants treated with ouabain and 40 min in

controls. Then, stomata partially closed for 1 h and opened to the same extent as reference plants. In the presence of ouabain the transpiration rate (Fig. 2*B*) was increased by about 75 % compared with controls.

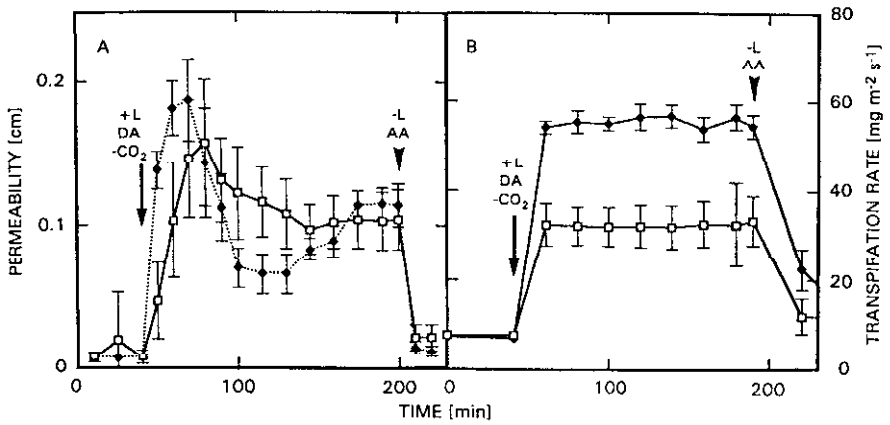

Fig. 2. Coefficient of stomatal permeability (*A*) and transpiration rate (*B*) of rye in absence (*squares*) or in presence (*rhombs*) of 10^{-5} M ouabain as a function of time. *Arrows* on graphs denote times at which shoots were submitted to the new environmental conditions. (+L - light on; –L - light off; DA - dry air; AA - ambient moist air; $-CO_2$ - CO_2-free air) *Vertical bars* = + S.E.

CO_2 removal in darkness: In darkness, stomata of control plants were closed when the leaf was swept by ambient air or dry air (Fig. 3*A*), then removal of CO_2 induced within 10 min a partial stomatal opening. Light application immediately promoted opening to a maximum within 30 min. In darkness and CO_2-free air the stomatal permeability was about 31.5 % of the potential maximum one under light. The presence of ouabain in root medium enhanced stomatal opening (+178 %) and opening was quicker (Fig. 3*B*). Light supply induced a wider aperture (+57 %) than that obtained without ouabain. Then, in the absence or in the presence of ouabain, stomata slightly closed to a steady state. Stomatal closure was quickly obtained with ambient air in darkness.

The time course of leaf transpiration rate was similar to that of the stomatal conductance under the same conditions (Fig. 3*C*). In darkness and dry air without CO_2, the transpiration rate reached about 60 % of that obtained under light and CO_2-free air. The presence of ouabain (Fig. 3*D*) enhanced transpiration rate in darkness and under light and CO_2-free air, in comparison with control plants.

CO_2 removal in light: On leaves subjected to a flow of ambient air, light supply induced a partial stomatal opening: 36 % of the potential one (Fig. 4*A*). Removal of water vapour in air hardly affected stomatal conductance but CO_2 removal promoted stomatal opening. Stomatal aperture in the presence of ouabain (Fig. 4*B*) was wider when the light was put on (+50 %) and when CO_2 was removed in the light (+14.3 % in relation to control plants). CO_2 and dry air enhanced transpiration rate of control plants (Fig. 4*C*). In the presence of ouabain (Fig. 4*D*), the curve of transpiration rate

looked very similar in shape to the previous one but values were higher, from +31 to +60 % depending on external factors.

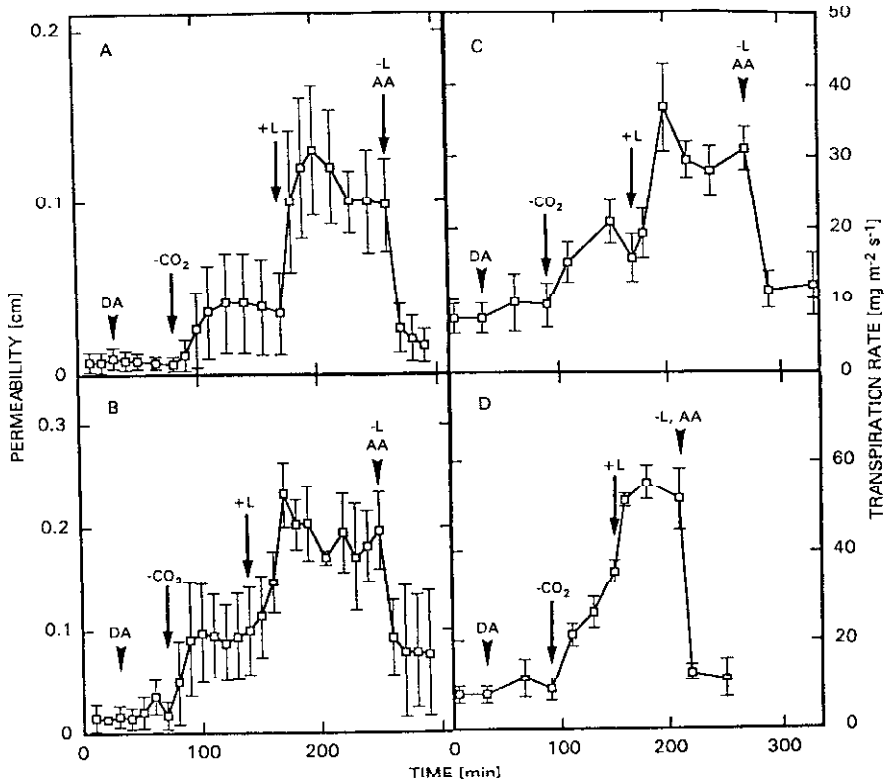

Fig. 3. Effect of the absence of CO_2 in darkness and in light on the coefficient of stomatal permeability (*A,B*) and transpiration rate (*C,D*) of rye as a function of time (*A,C* - control; *B,D* treated with 10^{-5} M ouabain). Symbols as in Fig. 2. *Vertical bars* = + S.E.

Discussion

[^{3}H]ouabain added into the root medium accumulated significantly in the leaves [15.6 nmol g^{-1}(f. m.) after 16 h of irradiance] which allow a possible direct action of ouabain in leaves and especially in guard cells.

In *Secale cereale*, stomatal opening was maximal when the leaf was submitted simultaneously to light and CO_2-free air. One of these effectors acting alone always induced a smaller opening. Stomata opened immediately when the plant was illuminated whereas stomatal response to zero CO_2 in darkness required about 10 min before opening occurred. When the leaf conductance was very low, at the beginning of the experiment in darkness, the response to CO_2-free air was almost zero.

Lascève *et al.* (1987) showed in *Zea mays* that CO_2-free air in darkness induced K^+ and Cl^- influx towards stomatal and subsidiary cells. Movements of ions were

recorded parallelly with the stomatal opening of *Vicia faba* and *Pelargonium* × *hortorum* (Laffray 1987). Opening in darkness and CO_2-free air was similar to that in light and ambient air. Ouabain inhibited Na^+ efflux from guard cells without disturbing K^+ uptake (Cram 1968). Ions accumulation might induce an overopening. The mechanical reaction of cell walls to this excessive swelling might then provoke a partial stomatal closure. The transpiration rate was also enhanced in the presence of

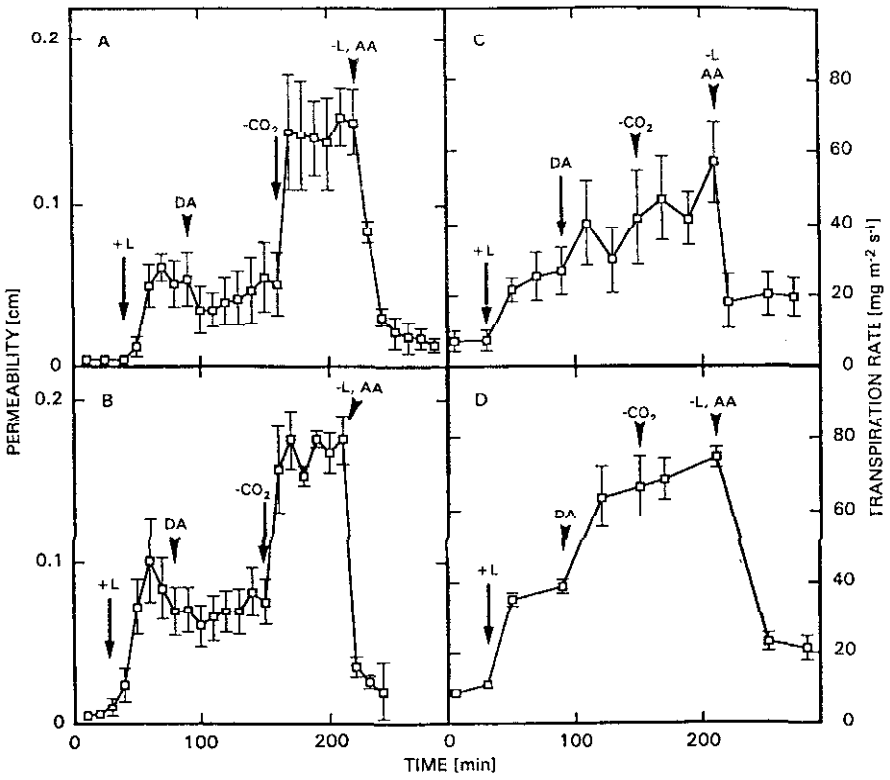

Fig. 4. Effect of the absence of CO_2 in light on stomatal conductance (*A,B*) and transpiration rate (*C,D*) of rye as a function of time (*A,C* - control; *B,D* - 10^{-5} M ouabain). Symbols as in Fig. 2. *Vertical bars* = ± S.E.

ouabain but without the same depression. Stomatal closure and cuticular transpiration (measured during the night) were not affected by the presence of ouabain. Our results seemed to show a possible role of Na^+ efflux in stomatal opening that would be associated with plasmalemma polarization or ionic pump activation. Sequential application of stimuli (Fig. 3 and 4) suggested that CO_2 removal and light would act on Na^+ efflux because stomatal apertures were larger in presence of ouabain in relation to control plants. The curve of transpiration rate looked similar in shape to that for stomatal permeability but values stayed low in darkness and reached a high level only in light. As in *Tradescantia virginiana* (Nonami and Schulze 1989), our

results showed that transpiration rate and stomatal conductance were not related to each other linearly. Na^+ efflux would be either involved in the initial stomatal opening processes because in the presence of ouabain the velocity and the degree of stomatal aperture are more important or the energy not used for inhibition of Na^+ efflux would be available for transport of other more efficient osmoticum. If ouabain reduced soluble saccharides translocation as in sunflower (Shiroya and Kura 1979), then ions would contribute mainly to the osmotic buildup required for stomatal opening movement.

In light and ambient air Na^+ ions would play a regulatory role on plasmalemma polarization (inducing permeability variations), enzymatic activities or osmotic potential.

Our results on a whole plant are inconsistent with those of Thomas (1970) obtained with epidermal strips. As it was previously mentioned, it is difficult to extrapolate from an observation made with epidermal peels to stomatal reponses in intact leaf (Zeiger 1983, Curvetto and Delmastro 1990).

To test whether ouabain realy inhibits Na^+ efflux out of guard cells as in animal cells, ionic analysis might be realized during stomatal movements. Also investigations on guard cell protoplasts in presence of ouabain might enable to a better understanding its role in plant cell membranes.

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