Regulation of a plant plasma membrane sucrose transporter by phosphorylation

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Abstract The protein phosphatase inhibitor okadaic acid (OA) either provided directly to sugar beet (*Beta vulgaris* L.) leaf discs or infiltrated in the leaf blade rapidly inhibited sucrose uptake. Methyl okadaic acid, a biologically inactive analogue of OA, had only a marginal effect on uptake. OA inhibited proton-motive force-driven uptake of sucrose into plasma membrane vesicles, without affecting their proton permeability. OA did not significantly affect the amount of sucrose transporters present in the vesicles, as estimated by ELISA with specific antibodies. It is concluded that OA directly inhibits the activity of a H^+-sucrose cotransporter of the plant plasma membrane, likely by maintaining it in a phosphorylated form.

Key words: Assimilate transport; Membrane phosphorylation; Okadaic acid; Plant; Plasma membrane; Sucrose transporter

1. Introduction

Physiological evidence indicates that plant cells may absorb reduced organic compounds via proton-coupled symporters which are specific for sucrose [1–4], hexoses [2,5,6], amino acids [7,8], small peptides [9–11] and glutathione [12]. In vivo, the activity of these transporters depends on the pmf which is created by a plasma membrane H^+-ATPase. In vitro, their activity may be directly characterized without interference of the ATPase by using PMV that are energized by an artificial pmf [3,4,8,13]. The cloning of various sucrose [14,15], hexose [16,17], amino acids [18–20] and peptide [21,22] transporters has resulted in the availability of molecular probes allowing the study of their expression at the transcriptional level. Specific antibodies directed against sucrose transporters have been prepared, allowing the study of their localization in different cell types [23,24] and the estimation of their amount in PMV [25,26]. Overall, this provides a favorable background to study the regulation of transport activity when the plant cells have to face various challenges including attacks by phytopathogens or mechanical wounding.

Available studies have mainly focused at the regulation of transporter expression at the transcriptional level, and show that elicitor treatment or mechanical wounding may increase the activity of the H^+-ATPase by transcriptional and post-translational controls [27,28]. Evidence has also been given for transcriptional [26,29] and post-translational regulation [26] of sugar transporter activity. One of the most common types of post-translational regulation of protein activity involves phosphorylation/dephosphorylation. Major plasma membrane primary pumps (H^+-ATPase [27,30]), channels (plasma membrane integral protein [31]) and major enzymes of sugar [32] and nitrogen [33] metabolism are controlled in this way. However, this type of regulation has not yet been demonstrated for proton-coupled plasma membrane transporters. The present paper demonstrates that sucrose transport activity of the plant plasma membrane is directly affected by OA, a protein phosphatase inhibitor, which suggests that phosphorylation of the sucrose transporter(s) inhibits its (their) activity.

2. Materials and methods

2.1. Plant material

Sugar beet (*Beta vulgaris* L. var. Aramis) were grown as described in [34]. Mature exporting leaves (4–5 weeks old) were used for all the experiments described below.

2.2. Transport assays in vivo

The uptake capacity of leaf discs was studied essentially as described in [35]. Briefly, leaf discs (6 mm diameter) were excised from the leaves after peeling of the lower epidermis, and incubated (peeled face downwards) on a basal medium containing 300 mM mannitol, 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, 20 mM MES (pH 5.5), added with 1 mM [U-^{14}C]sucrose (11 kBq ml^{-1}), 1% DMSO ± 1 μM OA. After incubation with the labelled substrate (30 min), apoplastic label was removed by three washes of 3 min each on basal medium. The discs were digested and their radioactivity was counted by liquid scintillation spectroscopy. OA and MOA were obtained from Coger (Paris, France).

2.3. Leaf infiltration

In some experiments, OA was infiltrated in the leaves before preparing the leaf discs or purifying the PMV. For this, the petioles of the leaves were dipped in 20 ml medium containing 2.5 mM MES (pH 5.5), 1% DMSO ± 2 μM OA. The leaves were allowed to transpire for 2 h under Sylvania GroLux lights (3600 μmol m^{-2} s^{-1}). Leaf discs were then prepared as described above after peeling of the lower epidermis, and used for uptake assays. Purified PMV were prepared as described below.

2.4. Transport assays in vitro

Purified PMV were prepared according to [36], from the leaf blades of infiltrated leaves, after discarding the petioles. These PMV were used to measure pmf-driven uptake of sucrose as described in [4]. Briefly, the PMV were first equilibrated in a medium containing 0.3 M sorbitol, 50 mM potassium phosphate (pH 7.5), 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$ and 0.5 mM DTT, at a concentration of 15 μg protein ml^{-1}. They were resuspended in a medium containing 0.3 M sorbitol, 50 mM sodium phosphate (pH 5.5), 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, 0.5 mM DTT, 10 μM valinomycin and 1 mM [U-^{14}C]sucrose (27 kBq ml^{-1}). These conditions established an artificial pmf due to the combination of the pH gradient (pH 7.5 inside; pH 5.5 outside) and of the electrical gradient resulting from the diffusion of potassium

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Abbreviations: AC, accession code; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DMSO, dimethylsulfoxide; MOA, methyl okadaic acid; OA, okadaic acid; PM, plasma membrane; pmf, proton-motive force; PMV, plasma membrane vesicles

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outwards the vesicles in the presence of the potassium ionophore valinomycin. This allows a direct study of the activity of the plasma membrane cotransporters mediating H^+-coupled sucrose uptake, without interference of the plasma membrane H^+-pumping ATPase which normally energizes this process in vivo, and without metabolism.

2.5. Proton-pumping and H^+-ATPase activity

Vanadate-sensitive ATPase activity of the PMV was measured as described [28]. Proton pumping was measured by the decrease of 9-aminoacridine absorbance at 495 nm [28].

2.6. Immunological methods

The amount of sucrose transporter present in the PMV was estimated by ELISA as described in [26] using a rabbit polyclonal antiserum directed against a sucrose transporter from *Arabidopsis thaliana* (AtSUC1, [37]).

3. Results

The possibility that the activity of the plasma membrane sucrose transporter is controlled by its phosphorylation status was tested by studying the effects of okadaic acid, a toxin affecting preferentially PP1 and PP2A protein phosphatases [32]. Fig. 1 describes the effects of OA and of its metabolically inactive analog MOA on sucrose uptake by peeled sugar beet leaf discs. When the treatment was conducted directly on peeled leaf discs (Fig. 1A), sucrose uptake was inhibited 25% and 44% by a 30 min and 2 h pretreatment with OA respectively, whereas MOA had no significant effect.

Although the conditions of treatment used in the present study are relatively shorter and milder than those usually described (see for example, [38,39]), a number of direct or indirect effects of OA on cell metabolism, on the PM H^+-ATPase, and on the sucrose transporter itself may explain these data. To investigate in more detail the cause of this transport inhibition, it was necessary to test the effects of OA at the membrane level with purified PMV. However, the preparation of purified PMV requires a large amount of plant material, and it is not possible to peel the epidermis of many sugar beet leaves within a reasonable lapse of time. To circumvent this problem, a method of infiltration via the transpiration stream was used (see Section 2). In order to check the effects of OA supplied via the transpiration stream, leaf discs were excised, peeled, and used for uptake assays in the usual way. Fig. 1B indicates that sucrose uptake is inhibited 31% and 44% respectively after infiltration with 2 μM OA for 1 and 2.5 h, compared to the corresponding controls. MOA only slightly affected sucrose uptake under similar conditions. These data indicate that infiltration with 2 μM OA allows a good distribution of the inhibitor in the leaf tissues and reproduces the effects of direct treatment with 1 μM OA on leaf discs. Therefore, for biochemical studies, purified PMV were prepared either from control leaves or from leaves infiltrated for 2.5 h with 2 μM OA.

Vanadate-sensitive ATPase activity and proton-pumping

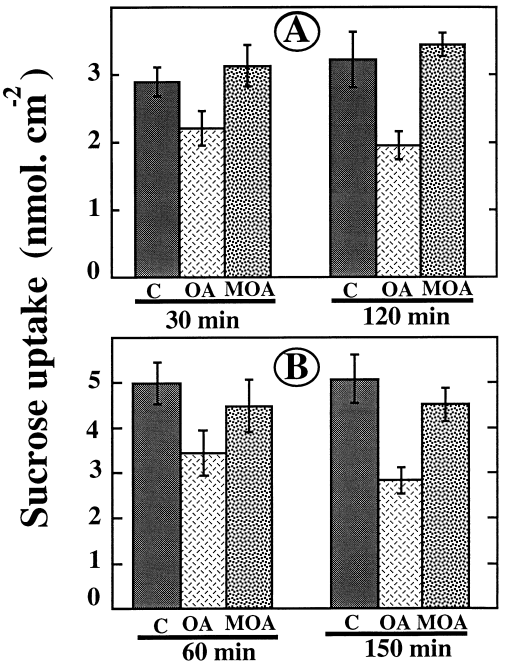

Fig. 1. Effects of OA and MOA on sucrose uptake by sugar beet leaf discs. In A, the discs were peeled and floated directly on 1 μM OA (or MOA) for the indicated time before the uptake assay (30 min). Data are means of 20 discs ± S.E. (from two independent experiments). In B, the leaves were infiltrated for the time indicated with 2 μM OA (or MOA), before the preparation of leaf discs and the uptake assay. Data are means of 80–100 discs ± S.E. (from four independent experiments).

activity of PMV from OA-infiltrated leaves were not significantly different from that of PMV from control leaves (Table 1). This indicates that the proton permeability of the vesicles was not affected by OA.

In contrast, pmf-driven uptake of sucrose was strongly reduced in PMV prepared from OA treated leaves (Fig. 2). Treatment with the protonophore CCCP shows that OA had hardly any effect on the pmf-independent uptake (Fig. 2). Therefore, inhibition by OA concerned the pmf-driven component of uptake.

The amount of sucrose transporters present in PMV from control and OA-infiltrated leaves was estimated by ELISA with a serum directed against the AtSUC1 sucrose transporter. Western blot analysis of purified sugar beet PMV has shown that this serum recognizes a single band at 42 kD [26]. Previous biochemical approaches developed in our lab had already identified a 42 kD band of sugar beet plasma membrane as a putative sucrose transporter [34,40,41]. It is therefore reasonable to conclude that the protein recognized by the anti-AtSUC1 serum reacts with a sugar beet sucrose transporter. The amount of material recognized by this serum

Table 1
Effects of OA on proton pumping and vanadate-sensitive activity of PMV

Treatment	Vanadate-sensitive ATPase activity (nmol Pi mg protein^{-1} min^{-1})	Proton pumping (OD units mg protein^{-1} min^{-1})
Control	185 ± 39	0.19 ± 0.02
OA	202 ± 35 (NS)	0.22 ± 0.03 (NS)

PMV were prepared from leaves infiltrated for 2.5 h with or without 2 μM OA. Data are means of 9 (ATPase) or 16 (proton-pumping) measurements from three independent PMV preparations ± S.E. (NS, non-significantly different from the control at $P = 0.95$ with the Student-Fisher test).

in the ELISA assay was not significantly different in PMV from control (0.891 ± 0.027 OD mg protein^{-1}, mean ± S.E., 15 measurements from three independent experiments) and treated (0.837 ± 0.029 OD mg protein^{-1}, mean ± S.E., 15 measurements from three independent experiments) leaves, suggesting that OA does not affect the synthesis and targeting of the sucrose transporter in the PM.

Previous work has shown that the sucrose uptake component which is induced by ageing was poorly sensitive to the non-permeant thiol reagent PCMBS [35]. This led us to study the effects of PCMBS after OA treatment. Without infiltration with OA, PCMBS inhibition of sucrose uptake reached 68% of the control (Table 2; PCMBS vs. control). In OA-infiltrated tissues, PCMBS inhibition reached only 51% (Table 2; OA+PCMBS vs. OA). Treatment of the tissues both with OA and PCMBS did not result in any significant increase of the inhibition compared to PCMBS alone. This shows that the OA inhibited component of uptake is entirely sensitive to PCMBS.

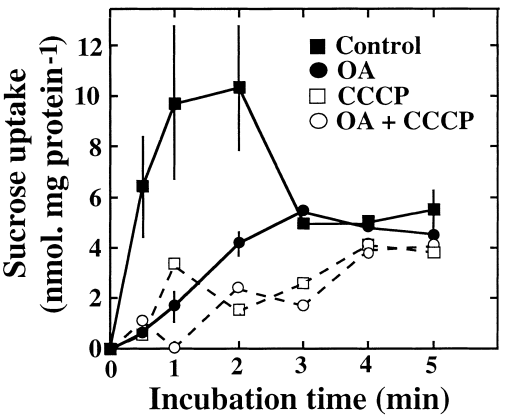

Fig. 2. Proton-motive force driven uptake of sucrose into PMV prepared from leaves infiltrated with or without 2 μM OA for 2.5 h. Data are means of four replicates and the experiment was repeated two other times with similar results. Some data points are shown ± S.E.

4. Discussion

The activity of major enzymes controlling uptake of organic compounds from the external medium, i.e. the PM H^+-ATPase and the H^+-coupled sugar and amino acid transporters must be tightly regulated in plant and yeast cells. The presence of glucose in the medium activates the yeast PM H^+-ATPase at the transcriptional level [42] and at the post-translational level by phosphorylation [43]. In case of phytopathogen attack, the activity of the plant PM H^+-ATPase is strongly stimulated by elicitors, via a phosphorylation process [27]. However, other data suggest that this enzyme may be activated directly or indirectly by dephosphorylation [33,44].

Bacterial and fungal elicitors also increase the amounts of transcripts encoding Stp4, a plasma membrane hexose transporter [29]. Cutting and ageing control the activity of the PM H^+-ATPase and of the sucrose transporters at the transcriptional and post-translational levels [26,28]. Thus, ageing induces an increase in the amount of transcripts encoding the sucrose transporters, and an increase in the amount of sucrose transporter protein present in the PM. However, this is not accompanied by an increased activity of the transporters as measured with PMV energized by an artificial pmf [26]. These data suggested that some kind of post-translational control is exerted over the activity of the sucrose transporter(s).

Antisense repression of sucrose transporter in phloem cells under the control of rolC promoter did not affect pmf-driven sucrose uptake in PMV, whereas ubiquitous repression of the transporter under the control of the CaM35S promoter strongly decreased pmf-driven sucrose uptake in PMV [25]. It may be concluded that uptake measured in PMV mainly reflects the activity in non-phloem cells. Although different sucrose transporters have been described in several species (for example AtSUC1 and AtSUC2 in *A. thaliana* [15], PmSUC1 and PmSUC2 in *Plantago major* [23]), no data are available for sugar beet, where one or several sucrose transporter(s) may be present. As a consequence, the data presented here refer to a sucrose transporter expressed at least in the mesophyll parenchyma cells, which represent the main source of PMV in whole leaf extracts. This excludes neither that this transporter is also expressed in phloem cells nor the possible existence of other transporters which may be not sensitive to (de)phosphorylation.

The present paper indicates that post-translational control of a sugar beet sucrose transporter involves its phosphorylation status. Indeed, the protein phosphatase inhibitor OA markedly inhibited sucrose uptake into leaf discs, whereas the analog MOA had only marginal effects (Fig. 1). Further experiments with PMV show that OA affects the pmf-driven component of sucrose uptake (Fig. 2), without affecting the proton-pumping ATPase activity nor the proton permeability of the PMV (Table 1). This indicates that the inhibition of sucrose uptake in leaf discs and in PMV directly concerns the amount and/or the activity of the transporter itself, and does not result from modifications of the pmf across the membrane. Since OA does not significantly affect the amount of material immunoreacting with a polyclonal serum directed against the *A. thaliana* sucrose transporter, these data strongly suggest that the main effect of OA concerns the activity of a sucrose transporter, and that dephosphorylation activates this protein. The OA-sensitive component of sucrose uptake is also sensitive to PCMBS, which is in good agreement with the known properties of sucrose transporters, and confirms that the OA effect directly concerns a transporter. Computer analysis of the peptide sequence of the sugar beet sucrose transporter (BvSUT1, AC 83850) reveals the presence of several serine and threonine residues located in cytoplasmic fragments and in consensus sequences for potential phosphorylation. Alignments with other sucrose transporter sequences (AtSUC1, AC X75365; AtSUC2, AC X79702; PmSUC1,

Table 2
Effects on OA and PCMBS on sucrose uptake by sugar beet leaf discs

Treatment	Uptake (nmol cm^{-2})
Control	9.03 ± 1.02[a]
OA	5.16 ± 0.51[b]
PCMBS	2.88 ± 0.54[c]
OA+PCMBS	2.52 ± 0.48[c]

The leaves were infiltrated with buffer or 2 μM OA for 2.5 h, and leaf discs were excised and floated on 1 mM [^{14}C]sucrose for 30 min in the absence or in the presence of 2 mM PCMBS. Data are means of 25 discs ± S.E. The experiment was repeated two other times with similar results. Numbers followed by different letters are significantly different ($P = 0.05$).

AC X84379; PmSuc2, AC X75764; NtSUT1, AC X82277; LeSUT1, AC X82275; SoSUT1, AC Q03411; StSUC1, AC X69165; VfSUT1, AC VFZ93774) indicate that some of these residues are highly conserved (Ser^{38}, Ser^{99}, Thr^{103}, referring to BvSUT1 sequence). Further experiments are presently underway to identify the amino acid residues that might be involved in the control of activity by phosphorylation. It has recently been shown that cutting may activate kinases in plants [45], and that sugars may increase protein kinase activities associated with the plasma membrane [46]. Our data suggest that a plant plasma membrane sucrose transporter is less active under its phosphorylated form. This regulation might for example allow a weaker retrieval of exogenous sugars by mesophyll cells in case of high intracellular sucrose concentration. The signals (light, sugar status, wounding) and the transduction pathways involved in the possible regulation of sucrose transporter activity by phosphorylation remain to be investigated.

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