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Method

In vivo assay to monitor flavonoid uptake across plant cell membranes

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ABSTRACT

Flavonoids represent one of the most important molecules of plant secondary metabolism, playing many different biochemical and physiological roles. Although their essential role in plant life and human health has been elucidated by many studies, their subcellular transport and accumulation in plant tissues remains unclear. This is due to the absence of a convenient and simple method to monitor their transport. In the present work, we suggest an assay able to follow *in vivo* transport of quercetin, the most abundant flavonoid in plant tissues. This uptake was monitored using 2-aminoethoxydiphenyl borate (DPBA), a fluorescent probe, in non-pigmented *Vitis vinifera* cell cultures.

1. Introduction

Flavonoids are a group of plant polyphenolic secondary metabolites with a common three ring chemical structure (C_6–C_3–C_6, named A, C and B ring, respectively). The major classes of flavonoids are anthocyanins (red to purple pigments), flavonols (colourless to pale yellow pigments), flavanols (colourless pigments that become brown after oxidation), and proanthocyanidins or condensed tannins. These compounds are widespread in plants, in various amounts, dependent on the plant species, organ, developmental stage and growth conditions. They are involved in several physiological functions, such as antioxidant activity, UV-light protection, defence against phytopathogens, regulation of legume nodulation, male fertility, pollinator attraction and control of auxin transport [1].

Flavonoids play all these crucial roles thanks to a finely regulated transport and accumulation system allowing entrance into different subcellular compartments. Nevertheless a comprehensive view of the phenomenon has not yet been proposed and the process is still under investigation. The chemical nature of flavonoids and their activities depend on their structural class, degree of hydroxylation, other substitutions and conjugations. Spectral characteristics of most flavones and flavonols show two major absorption bands: Band I (320–385 nm) represents the B ring absorption, while Band II (250–285 nm) corresponds to the A ring absorption [2]. It has to be stressed that several flavonoids behave as pH indicators and their spectra could be strongly affected by the pH value of the assay medium [3]. This feature limits the utilization of the usual spectrophotometric and spectrofluorimetric transport assays.

Quercetin (QC) is a widely distributed natural flavonoid, found in many fruits, leaves and grains. It is able to modulate cell proliferation and apoptosis in different human cells. A high amount of QC is contained (values indicated in parentheses as mg/100 g FW) in the leaves of green (256) and black tea (205), cowberry (21), cranberries (14), apples (4) and red grapes (4) [4]. It is generally known that QC, like most other flavonoids, is poorly fluorescent in aqueous solutions, but exhibits an increased fluorescence when bound to specific probes such as 2-aminoethoxydiphenyl borate (DPBA) [5].

DPBA is an esterified moiety commonly used in microscopy for flavonoid localization [6], able to form a spontaneous complex with most represented flavonoid compounds [7]. Accordingly, it has already been used in tissue microscopy analysis to describe flavonoid accumulation inside specific plant compartments [8,9]. Similarly to flavonoids, the fluorescence of the probe/ligand complex seems to be very sensitive to the assay buffer and pH [10].

The present work was aimed at assessing a new methodological protocol to follow *in vivo* flavonoid transport, utilizing DPBA as a probe, in non-pigmented cell (NPC) cultures from *Vitis vinifera*, grown in the dark, which represent a reliable and easy-to-manage experimental model.

Abbreviations: DPBA, 2-aminoethoxydiphenyl borate; MS, Murashige and Skoog; NPC, non-pigmented cell; PFA, paraformaldehyde; QC, quercetin

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2. Materials and methods

2.1. Cell culture

Grapevine cell cultures (*Vitis vinifera* L. cv. Limberger) were grown on solid Murashige and Skoog medium (MS) [11] for 21 days and incubated at 25 °C in the dark, as described by Repka et al. [12]. Seven-day-old cultured cells were used for the uptake experiments.

2.2. Reagents

All reagents were purchased from Sigma–Aldrich. DPBA and QC were solubilized in pure ethanol to reach the desired concentration.

2.3. Spectrofluorimetric analysis of QC uptake in NPC cultures by DPBA

Three grams (FW) of 7 day-old cells were mixed in 20 ml of fresh MS medium and shaken to disrupt all the clusters. During the experiments, cells were maintained at constant shaking (110 rpm), at room temperature and shaded to avoid light exposure. Aliquots of the cell suspension were incubated with DPBA for 20 min, at a final concentration of 0.05% (w/v), corresponding to 2.2 mM (exceeding the QC concentrations used). The cells were then washed by centrifuging at about 400 *g* for 2 min, using a 50 μm nylon gauze, to eliminate all the liquid fraction. After filtration, the cells were collected by a gauze, resuspended in the same starting volume of fresh MS medium and maintained at constant shaking until the fluorimetric assay. Cells were then diluted (1:3 v/v) with fresh MS medium just before the analysis with spectrofluorimeter (Perkin Elmer, model LS50B). The reaction was started by addition of different concentrations of QC (ranging from 0.1 to 50 μM). The reading set up was: 461 nm for the excitation and 520 nm for the emission, using slit width of 5 and 10 nm for excitation and emission, respectively.

Transport inhibition was performed by incubating cells, preloaded with 0.05% DPBA for 10 min, with 0.1 or 0.3% (w/v) of paraformaldehyde (PFA) for further 10 min. The cells were then washed as described above and the inhibitory effect could be evaluated by comparing the initial rate of treated and untreated samples after the addition of 0.5 μM QC.

Sonication treatment, using 1 pulse for 20 s with 400 W of power, was applied to disrupt cell wall and cell integrity, to obtain vesicles more easily prone to detergent action. The protocol differed slightly from the one proposed by Pereira-Lachataignerais et al. [13] because one single pulse was sufficient to disrupt the cell wall and necessary to obtain bigger vesicles able to be kept into the nylon gauze. After sonication, the vesicles were washed and resuspended in a double volume of starting MS medium, and subsequently diluted (1:3 v/v) in MS medium, before the spectrofluorimetric analysis. SDS (0.3% w/v, final concentration) was added to allow the release of DPBA from the cells.

2.4. Total QC evaluation in cell extracts by DPBA

The analysis was performed according to the method described by Lee et al. [7], with minor changes. Briefly, 4 g (FW) of cells were diluted in 16 ml of MS medium, split into four 4 ml-flasks and then incubated for 1 h at different QC concentrations (0, 0.5, 5 and 50 μM). Cells were collected and washed as reported previously. The filtered cells were resuspended in 4 ml of cold methanol and, after disruption by 3 pulses of sonication for 20 s at 400 W, the homogenates were incubated overnight in the dark at room temperature and at constant shaking. Two centrifugations at 27,000 *g* for 5 min were performed in sequence on the supernatant to discard all the cell debris. A continuous nitrogen flux was used to dry the solvent and the powder was resuspended in 400 μl of cold methanol.

DPBA (0.05%, w/v) and samples were mixed 1:2 and fluorescence signal was measured using a Multilabel Counter (WALLAC, model 1420, Perkin–Elmer) set at 465 ± 10 nm (excitation filter) and 535 ± 10 nm (emission filter), respectively.

3. Results

3.1. Emission spectra of DPBA and DPBA/QC complex

With the aim to determine the appropriate wavelength range for slit setting, the emission spectrum of DPBA and QC-bound DPBA was evaluated (Fig.1). The figure shows a rather large spectrum (between 500 and 590 nm), where the emissions of bound and unbound DPBA differ from each other. An emission value of 520 nm was, therefore, chosen for spectrofluorimetric analysis, according to Murphy et al. [14]. Instead, an emission value of 535 nm was used for the detection, using Multilabel Counter.

3.2. In vivo QC uptake in grapevine suspension cell cultures

The assay was carried out aiming to follow the QC uptake in a plant cellular system. Grapevine suspension cell cultures were loaded with DPBA (0.05% w/v) and then different QC concentrations (ranging from 0.1 to 50 μM) were added. The formation of the fluorescent complex between the probe and the flavonoid was demonstrated by a microscope under fluorescent light (Fig. 2). Panel a shows that, in the presence of DPBA only, grapevine NPC exhibited a low fluorescence, which strongly increases after the addition of QC (panel b). In agreement, during spectrofluorimetric assay cells showed rising fluorescence values at increasing concentration of the added flavonoid (panel c). When the initial rates of QC uptake into cells were plotted *versus* QC concentration, a hyperbolic regression curve, describing the kinetic features of *in vivo* QC uptake through the plasma membranes, was obtained. Both the washing step and the DPBA loading treatment did not alter the cell viability (data not shown).

3.3. Spectrofluorimetric analysis of DPBA/QC uptake

To verify if the complex DPBA/QC was not specifically bound to cell outer components and could be released by cells, suspension

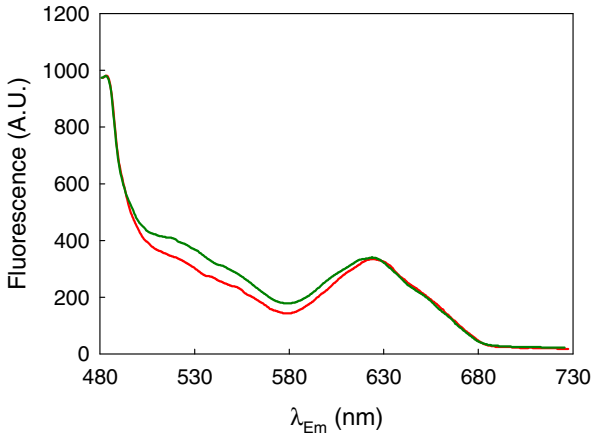

Fig. 1. Emission spectra of DPBA (red trace) and DPBA/QC complex (green trace) in MS medium. DPBA and QC were 0.05% and 0.5 μM, respectively.

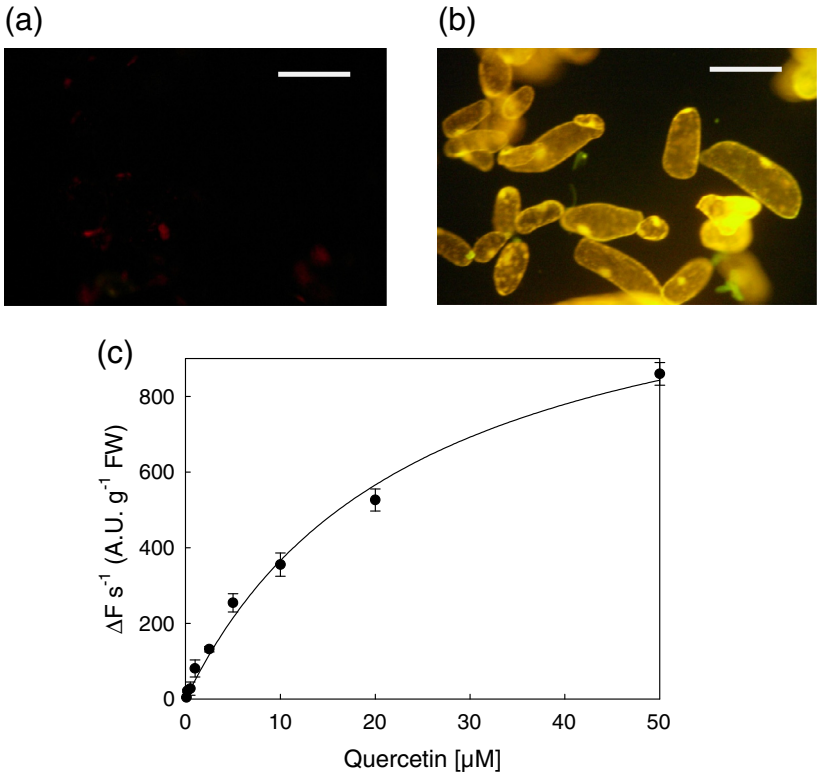

Fig. 2. Panels (a) and (b): images under fluorescent light of grapevine NPC cultures treated either with DPBA (a) or with both DPBA and QC (b). Scale bars = 50 μm. Panel (c): *in vivo* QC uptake in grapevine NPC cultures, measured by means of DPBA fluorescence. Values represent a mean of five independent replicates ± SD. Regression curve (single rectangular hyperbola) was obtained using the following equation: $y = a^*x/(b + x)$; a = 1250 ± 87 $\Delta F\ s^{-1}$ and b = 24.2 ± 3.5 μM.

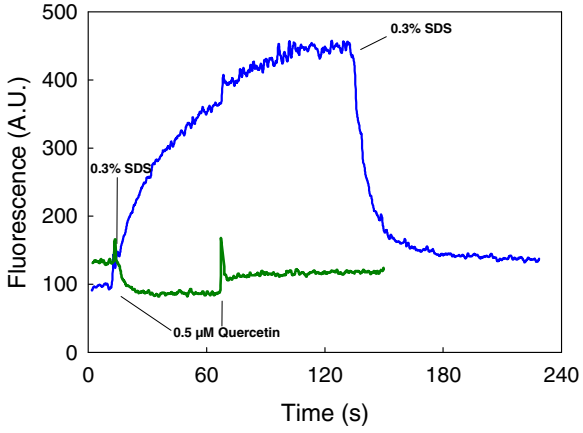

Fig. 3. Spectrofluorimetric analysis of DPBA/QC uptake in grapevine suspension cell cultures. Traces are representative of at least three independent replicates. Sonicated cells were used to check DPBA/QC movement within the vesicles, by firstly adding 0.5 μM QC (blue line) or 0.3% SDS (green line), respectively.

cell cultures were treated with DPBA, sonicated (to obtain vesicles with broken cell wall) and washed (Fig. 3). The addition of QC determined an increase of fluorescence, which was completely reversed by the subsequent addition of 0.3% SDS (blue trace). This suggests that the DPBA/QC complex entered the vesicles, to a greater extent when compared to intact cells. In agreement, the uptake was prevented if the vesicles were pre-treated with 0.3% SDS (green trace). However, the addition of SDS, before QC, still induced a small decrease in the fluorescence signal. This result indicates that vesicles already possessed a low, but detectable amount of endogenous flavonoids, of which the presence could be evaluated by DPBA.

Experiments performed with intact cells revealed the inability of SDS to solubilize plasma membranes, if the cell wall was still present (result not shown). SDS added to the assay was not able to alter the permeability of the cell wall in grape suspension cell cultures. Indeed, only after sonication and cell wall breaking, SDS effectively disrupted the phospholipid bilayer, thus allowing the release of the fluorescent complex DPBA/QCQuercetin. The phenomenon was visualized by fluorescence quenching of DPBA/QC complex, caused by its dilution into assay medium.

3.4. PFA inhibition of QC transport in grapevine suspension cell cultures

PFA is the smallest poly-oxymethylene (the polymerization product of formaldehyde), largely used in microscopy to fix the cells to preserve the membrane integrity. The PFA's aldehyde group can form a methylene bridge (–CH2–) between two reactive atoms of a protein. The reaction involves nitrogen and another reactive atom very similar to it in protein structure. Therefore, PFA has proven to be an excellent protein cross-linker, maintaining membrane structure and thus cellular compartmentation. To verify if the QC transport could be a protein-mediated phenomenon, the uptake of QC was studied after the addition 0.1 and 0.3% PFA to the assay medium (Fig. 4). After DPBA loading and incubation with different PFA concentrations, QC uptake was evaluated as the initial rate of fluorescence increase. In the presence of PFA, the uptake showed an inhibition of more than 30% (0.1% PFA, ΔF/min = 299) and more than 70% (0.3% of PFA, ΔF/min = 86), when compared to the control (ΔF/min = 583). This result supports and confirms the hypothesis that QC could be transported by membrane proteins. Accordingly, DPBA transport, occurring during previous loading, was also

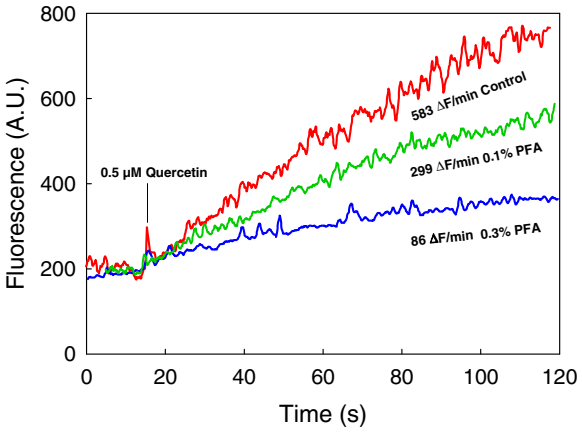

Fig. 4. PFA inhibition of QC transport in grapevine suspension cell cultures. The percentages of PFA used for conditioning grape suspension cell cultures were, respectively, 0% (blue line), 0.1% (green line) and 0.3% (red line). Each curve is representative of three different experiments.

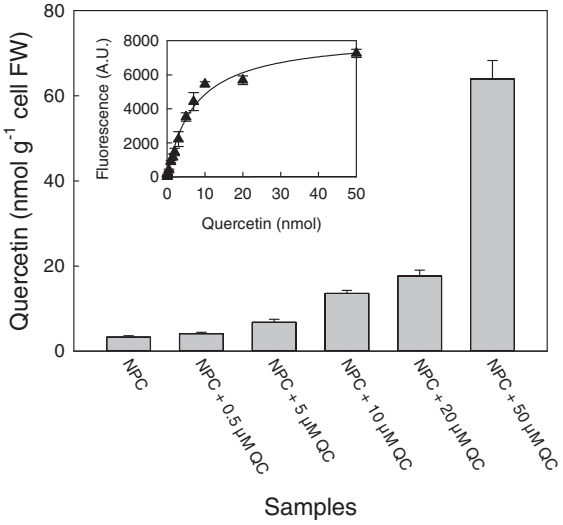

Fig. 5. QC determination in cell methanolic extracts. Different QC concentrations (0.5, 5, 10, 20 and 50 µM, respectively) were added to grapevine suspension cell cultures. The inset plot shows the calibration curve of fluorescence intensity obtained with different amounts of QC, ranging from 0 to 50 nmol. Values are means of three independent replicates ± SD.

slowed by the PFA treatment, indicating again the possible involvement of a protein-mediated transport (data not shown).

3.5. QC quantification in cellular extracts

The actual amount of QC taken up by the cells was quantified by DPBA reactions in methanolic extracts. The corresponding fluorescence values were converted by means of a calibration curve constructed using known QC concentrations. As shown in Fig. 5, NPC cultures without any QC addition contained a detectable amount of approx. 4 nmol/g FW of endogenous flavonoids, which then doubled (10 nmol/g FW) in the occurrence of incubation with 5 µM of QC. When incubated with a large concentration of the flavonoid (50 µM), the cells were able to accumulate a greater amount, reaching a level of approx. 71 nmol/g FW. Considering that the concentration of 50 µM QC corresponds to 200 nmol of QC g^{-1} FW of cells, it can approximately be estimated that cells were able to take up to 30% of the added QC in the assay medium.

4. Discussion

Currently, methodologies to monitor plant flavonoid transport *in vivo* are lacking, since spectrophotometric and fluorimetric assays are not easily applicable to these molecules. Previously, the transport of flavonoids in plant cells was evaluated with regard to the accumulation of these metabolites in the cells. To do this, analytical [15], biochemical [16,17] and indirect genomic approaches [18] were applied, mainly based on flavonoid quantification and identification by HPLC.

For these reasons, the fluorimetric method described in this paper can be viewed as a new tool useful to study flavonoid uptake into *in vivo* plant cells. The dye (DPBA) has been originally used for histological analysis of flavonoids by microscopic techniques, but recently Lee et al. [7] applied it also for rapid quantification of flavonoids in methanol extracts from animal cells. The method here described allows the detection and characterization of biochemical parameters linked to flavonoid transport into plant cells, an uptake probably mediated by membrane carriers. This protocol appears to be reliable even in the presence of cell wall, which could be an interfering structure in the case of plant systems. Indeed, it seems to be stable during long-lasting experiments, allowing an easy-to-use, fast and, moreover, direct method to evaluate flavonoid accumulation.

The spectrum analysis of QC-bound and free DPBA in a cell-free medium revealed that the complex shows high emission fluorescence, ranging from 500 to 600 nm, which allows a wide selection of the proper analytic setup (Fig. 1). After the loading of DPBA in NPC of grapevine and the washing of external dye in excess (Fig. 2, panel a), cells exhibited an increase of fluorescence intensity when QC was added (Fig. 2, panel b). The signal was confined within the cells and, in particular, was localized in the nucleus and cytoplasm, in agreement with Saslowsky et al. [8].

In addition, Fig. 2 panel c shows that the probe was not yet saturated by QC concentration (50 µM), thus permitting a good flexibility and a wide range of detection.

In previous papers, it was shown that pH conditioning is essential, according to the reversible chemical transformation of phenolic compounds under different pH conditions [19], and that the fluorescence increase of DPBA/flavonoid complexes are related to increasing pH [20]. This pH-dependent reaction, possibly due to DPBA/flavonoid complex entry in the cell, could explain the increase in fluorescence, detected in grapevine suspension cell cultures, which could reflect a true kinetic within a living system and not an unspecific effect or artefact. In addition, Fig. 3 shows that, after cell integrity disruption by mild sonication and microsomal vesicle formation, the QC uptake was higher than that observed in intact cells. This difference can be explained by these observations: i) the small volume of vesicles formed during sonication affects the fluorescence signal intensity, particularly if one considers that a plant cell (with a diameter approximately around 100 µm) possesses a volume 8-fold larger than that of vesicles (calculated on the basis of mesh size of the 50 µm nylon gauze); such condition allowed DPBA to be more easily saturated by QC inside the vesicles; ii) during sonication, vesicles can directly and quickly internalize greater amounts of DPBA, present in the assay buffer, with respect to intact cells; iii) the microsomal membrane system represents an environment more freely permeable for the probe (dye) and flavonoids in comparison to the intact cell, where different compartments and metabolites could inhibit the uptake, thus decreasing the associated fluorescence. Moreover, the QC uptake seems to be a process mediated by protein(s), since increasing concentrations of PFA, a known protein-linker, were able to inhibit it (Fig. 4).

The percentage of QC taken up into the cell actually represents a little part of the entire QC added to the assay mixture (Fig. 5). The amount of QC capable of entering the cells was about 30% of the total amount supplied, namely more than a double, if compared to similar experiments in animal cell systems (12%) [7]. On the other hand, plant cell systems should be more efficient and specialized to translocate QC and secondary metabolites. In this system, the DPBA is also able to detect endogenous flavonoids, present at nano-scale concentrations, within NPC of grapevine, as demonstrated by the fluorescence, monitored even in the absence of any external QC addition (Figs. 3 and 5). These data, therefore, confirm that flavonoids could be detectable by means of DPBA fluorescence also in plant cell extracts, aligning with previous results obtained in mammalian cells [21], and suggesting that this method is highly sensitive.

Hence, this new methodology can be considered as a initial approach that could then be applied to study the uptake of other flavonoids [22]. Further steps could involve the assessment of experimental conditions suitable for different polyphenolic moieties. Another application field would be the use of this method in a number of cell systems, aiming at defining a more general protocol for different organisms. In particular, dietary flavonoids, which are known to be transported at low concentration in mammalian cells, could be the next suitable candidates for their uptake detection by the DPBA fluorimetric method, considering also their essential role on human health and cancer proliferation [23].

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AF and EB conceived the project, AF acquired the data, AF and EB analyzed and interpreted the data, AF, EB and EP wrote the paper, AV, CP and AB reviewed the manuscript.

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