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Research article

Di-4-ANEPPDHQ, a fluorescent probe for the visualisation of membrane microdomains in living *Arabidopsis thaliana* cells

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ABSTRACT

Cholesterol-enriched microdomains, also called lipid rafts, are nanoscale membrane structures with a high degree of structural order. Since these microdomains play important roles in dynamic cytological events, such as cell signalling and membrane trafficking, the detection and tracking of microdomain behaviours are crucial to studies on modern membrane physiology. Currently, observation of microdomains is mostly based on the detection of specific raft-resident constituents using artificial cross-link fluorescent probes. However, only a few microdomain-specific fluorescent dyes are available for plant cell biology studies. In this study, the photophysical properties of di-4-ANEPPDHQ were analysed. The use of confocal laser scanning microscope (CLSM)-based methods in the visualisation of microdomains in living cells of *Arabidopsis thaliana* was assessed. The results confirmed that the generalised polarisation (GP) method can be used to quantitatively visualise the membrane orders in live plant cells. This dye was found to have low cytotoxicity in plant root epidermal cells and root hairs. These findings suggest that di-4-ANEPPDHQ is an appropriate tool for the visualisation of microdomains in living plant cells.

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1. Introduction

The plasma membrane (PM) is an inhomogeneous fluid bilayer system composed of different components (Simons and Ikonen, 1997). Among these constituents, sphingolipids and cholesterol have self-associative characteristics, forming specific membrane regions with a high degree of structural order (Simons and Ikonen, 1997; Lingwood and Simons, 2010). These so-called membrane microdomains, or lipid rafts, provide docking areas for functional proteins having essential roles in numerous cellular and intercellular events, such as signal transduction, vesicular recycling, endocytosis and exocytosis (Malinsky et al., 2013). Previous studies have suggested that the stimulated movement, assembly and formation of these lipid microdomains could play significant roles in several cellular events (Ovečka et al., 2010; Li et al., 2012, 2013). For this reason, *in vivo* observation of the dynamic behaviour of microdomains is of great interest in cell biology.

Precise observations depend on technological advances and the choice of methodology. Biochemical identification of detergent-resistant membranes (DRMs) provided the original evidence for the existence of microdomains in plant cells (Mongrand et al., 2004; Borner et al., 2005). However, since the use of DRMs as a criterion to determine the components of microdomains has been questioned, microscopic observation of the microdomain structures is required to provide more compelling evidence (Tanner et al., 2011). Electron microscopic observations of fixed plant cells labelled with microdomain-specific antigens have been used to describe the localisation and size of membrane microdomains and the formation of associated endosomes (Raffaele et al., 2009; Li et al., 2012). Confocal laser scanning microscope (CLSM) observations of the fluorescence proteins (FPs)-tagged marker proteins, such as remorin-FPs and flotilin-FPs, have provided new methods to investigate the dynamic behaviour of microdomains in living plant cells (Raffaele et al., 2009; Li et al., 2012). Sterol-specific fluorescent probes are able to label the lipid components directly on the PM and endosomes. A few of these probes have already been reported in plant cell biology. For example, FM serial dyes can label the PM uniformly without selectivity to microdomains (Bolte et al.,

List of abbreviations: CLC, clathrin light chain; CLSM, confocal laser scanning microscope; DRM, detergent resistant membrane; FP, fluorescence proteins; GP, generalized polarization; MS, Murashige and Skoog; PI, propidium iodide; PM, plasma membrane; ROI, region of interest.

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2004; Jelínková et al., 2010).

Filipin is a widely accepted selective fluorescence dye for microdomains that works by selectively forming a filipin–sterol complex. However, its use in living cells is limited by its cytotoxicity and by the high concentration and long incubation time required for effective staining (Ovečka et al., 2010; Boutté et al., 2011). Thus, the development of new fluorescence probes is necessary for studies on plant cell biology.

Laurdan and di-4-ANEPPDHQ are two phase-sensitive membrane probes (Parasassi et al., 1997) that have been shown to respond specifically to lipid packing other than membrane-associated peptides (Dinic et al., 2011). The excitation wavelength of Laurdan in live tissue imaging is ~800 nm, so it requires a complex and expensive two-photon laser scanning system (Owen et al., 2011). Di-4-ANEPPDHQ was assumed to be an appropriate fluorescence marker for imaging the microdomains in living plant cells. As a naphthylstyryl–pyridinium dye (di-n-ANEPPDHQ), di-4-ANEPPDHQ was originally synthesised as a transmembrane voltage-sensitive dye consisting of the napthylstyryl–pyridinium chromophore from di-8-ANEPPS and the quaternary ammonium head group (DHQ) from the dienylstyryl–pyridium dye RH795 (Fig. 1A) (Obaid et al., 2004). Jin et al. (2005) found that the emission spectra of di-4-ANEPPDHQ exhibited a significant blueshift in the lipid-ordered phase compared to the disordered phase, suggesting that the dye could be used in the imaging of membrane microdomains. The optical characters of di-4-ANEPPDHQ in artificial membrane systems and animal cells are well established by fluorescence lifetime imaging (FLIM) and CLSM, and a widely accepted protocol for quantitative *in vivo* imaging has been published (Owen et al., 2006, 2011). However, few studies have mentioned the application of di-4-ANEPPDHQ to the visualisation of microdomains in plant cells (Roche et al., 2008; Liu et al., 2009). Verification of its potential side effects and specificities and characterisation of the optical characters in live cells should be performed as essential prerequisites.

In this study, we characterised the emission spectra of di-4-ANEPPDHQ when bound to the PM and endosomes in *Arabidopsis*. The toxicity of this dye was also evaluated, providing an experimental prerequisite for use of this probe in living plant cells. A processing algorithm based on CLSM images was used to confirm that generalised polarisation (GP) values can also quantitatively visualise membrane order degrees in plant cells.

2. Materials and methods

2.1. Plant materials

Seeds of *Arabidopsis thaliana* ecotype Columbia (wild type, WT) were surface-sterilised with mixed disinfectants, hydrogen peroxide (H_2O_2) and 85% ethanol for 45 s, and then washed five times with sterilised double-distilled water. Sterile seeds were grown on ½ Murashige and Skoog (MS) medium supplemented with 1% sucrose and 0.4% phytagel in petri dishes, and then vertically cultured at 20–22 °C under long-day conditions (16 h light, 8 h dark) for 3 days.

2.2. Di-4-ANEPPDHQ staining

Di-4-ANEPPDHQ was purchased from Invitrogen (cat. no. D36802). The stock solution of di-4-ANEPPDHQ [5 mM in dimethyl sulphoxide (DMSO)] was stored in a 200-μL microcentrifuge tube wrapped with aluminium foil at −20 °C. For di-4-ANEPPDHQ staining, 3-day-old seedlings were incubated in the staining solution (5 μM Di-4-ANEPPDHQ in ½ MS medium) for 5 min on ice and washed with cold ½ MS medium for 1 min.

In the plasmolysis experiment, *Arabidopsis* seedlings were treated with 650 mM sorbitol for 5 min. For propidium iodide (PI) staining, the seedlings were incubated with staining solution, washed and transferred to a glass slide with 100 μL or 75 μM ½ MS medium and covered with a coverslip.

2.3. CLSM observations and GP processing

An SP5 CLSM (Leica, Germany) was used for the imaging of di-4-ANEPPDHQ-labelled seedlings. The sample was excited using a 488-nm laser, and the detection ranges of the two channels were

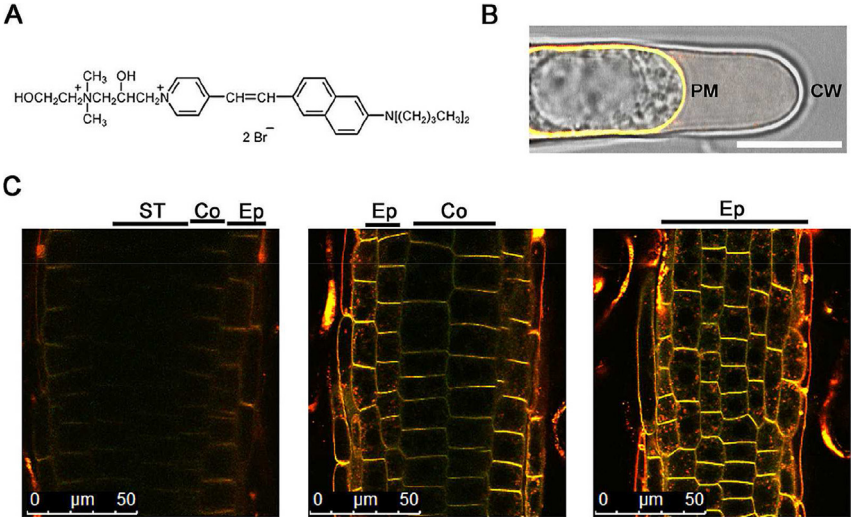

Fig. 1. Analysis of di-4-ANEPPDHQ emission profiles in intact plant cells. (A) Structural formula of di-4-ANEPPDHQ (product sheet from Invitrogen®). (B) Plasmolysis in root hair cells showed that di-4-ANEPPDHQ labelled the plasma membrane (PM) but not the cell wall (CW). Bar = 20 μm. (C) Optical sections of the di-4-ANEPPDHQ-labelled *Arabidopsis* root cells at the same position. Left: longitudinal section through the root stele (St); middle: longitudinal section through the root cortex (Co); right: longitudinal section through the root epidermis (Ep). Fluorescence signals in (B) and (C) were combined from dual channel images (red channel: 620–750 nm, green channel: 500–580 nm). The excitation laser wavelength was 488 nm. Images were selected from three independent experiments with at least three seedlings per replication. Bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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