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Short communication

Cycloheximide as a tool to investigate protein import in peroxisomes: A case study of the subcellular localization of isoprenoid biosynthetic enzymes

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ABSTRACT

Cytosolic background fluorescence is often observed when native low-abundance peroxisomal proteins carrying a weak peroxisomal targeting sequence are expressed as fluorescent fusion protein using a strong constitutive promoter in transiently transformed plant cells. This cytosolic fluorescence usually comes from the strong expression of the low-abundance proteins exceeding the peroxisome import efficiency. This often results in a misinterpretation of the protein subcellular localization, as there is doubt as to whether proteins are dually targeted to the cytosol and peroxisome or are exclusively localized to peroxisomes. To circumvent this experimental difficulty, the protein peroxisome import study can be optimized by *de novo* protein synthesis inhibition in transiently transformed cells using the translation inhibitor cycloheximide. This approach was used here successfully for the study of the subcellular localization of distinct plant isoprenoid biosynthetic enzymes, allowing us to clearly demonstrate that 5-phosphomevalonate kinase, mevalonate 5-diphosphate decarboxylase and a short isoform of farnesyl diphosphate synthase from *Catharanthus roseus* are exclusively localized to peroxisomes.
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Introduction

Peroxisomes are ubiquitous cell organelles that classically house oxidative metabolic reactions. In plants, peroxisomes participate in many physiological processes, including fatty acid β-oxidation, photorespiration, photomorphogenesis, as well as the synthesis of hormones or cell signaling by reactive oxygen species (Kaur et al., 2009). Such high metabolic activity relies on the diversity of proteins located to this subcellular compartment, as established by successive peroxisome proteome analyses (Reumann et al., 2007; Arai et al., 2008; Eubel et al., 2008; Reumann et al., 2009). This compartmentalization involves the peroxisome targeting of cytosol-translated proteins that contain a conserved peroxisome targeting type 1 (PTS1) or type 2 (PTS2) signal. PTS1 is a C-terminal tripeptide sequence corresponding to the canonical SKL sequence or its variants, while PTS2 is a nonapeptide sequence located at or near the N-terminus corresponding to the prototype RLX_5HL (Kaur et al., 2009). In a continuing effort to characterize the protein import into peroxisomes, studies combining proteomic and bioinformatic analysis have contributed to refining the PTS sequences, with a particular emphasis on PTS1 (Reumann, 2004; Reumann et al., 2007, 2009; Ma and Reumann, 2008; Lingner et al., 2011). For each new variant, functional validation is generally carried out by evaluating the ability of the proposed PTS to direct a fluorescent protein towards peroxisomes in transiently transformed plant cells expressing the fusion protein. However, such an approach can encounter technical limitations for low efficiency PTS (also called weak PTS) due to a strong and persistent fluorescence in the cytosol that masks peroxisome labeling. This phenomenon is amplified for low-abundance proteins that tolerate weak PTS for quantitative targeting to peroxisomes under native conditions, while the use of a constitutive promoter for the expression of the fluorescent fusion protein could exceed the peroxisome import efficiency. This often prompts researchers to extend the time of observation to more than one week to distinguish peroxisomes from the cytosolic background fluorescence (Lingner et al., 2011). Furthermore, this remaining cytosolic fluorescence prevents a conclusion that, in native conditions, proteins are exclusively localized in peroxisomes

Abbreviations: CHX, cycloheximide; CFP, cyan fluorescent protein; FPS, farnesyl diphosphate synthase; MVA, mevalonate; MVD, mevalonate 5-diphosphate decarboxylase; MVK, mevalonate kinase; PMK, 5-phosphomevalonate kinase; PTS, peroxisome targeting sequence; TDC, tryptophan decarboxylase; YFP, yellow fluorescent protein.

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or dually targeted to both the cytosol and the peroxisome. Such ambiguity has been recently exemplified by the compartmentalization studies of isoprenoid biosynthetic enzymes in *Catharanthus roseus*. In addition to the punctuated peroxisomal pattern, observed in transiently transformed cells, for 5-phosphomevalonate kinase (PMK), mevalonate 5-diphosphate decarboxylase (MVD) and farnesyl diphosphate synthase (FPS) fused to the yellow fluorescent protein (YFP), these proteins were also shown to display a diffuse cytosolic pattern (Simkin et al., 2011; Thabet et al., 2011). This prompted us to optimize the study of protein import into peroxisomes by testing the effect of *de novo* protein synthesis inhibition in transiently transformed cells, using the translation inhibitor cycloheximide (CHX).

Materials and methods

Plant cell material

All experiments were performed using *Catharanthus roseus* (Madagascar periwinkle) cell suspensions (C20A strain) propagated in Gamborg B5 medium (Duchefa) at 24 °C under continuous shaking (100 rpm) in the dark for 7 days. Prior to transformation, 4 ml of 3-day-old cell culture were vacuum filtered onto a 45-mm diameter circular piece of filter paper and plated onto solid Gamborg B5 medium (8 g l^{-1} agar) supplemented with 10 μM naphthalene acetic acid. Plated cells were then cultivated for 48 h in the dark at 24 °C before bombardment.

Constructs for subcellular localization studies

Constructs expressing the periwinkle tryptophan decarboxylase (TDC, Gb accession number M25151), the mevalonate pathway enzymes from *C. roseus*, mevalonate kinase (MVK, HM462019), PMK (HM462020), MVD (HM462021) and a short isoform of periwinkle FPS (HQ316638) fused to N-terminus or C-terminus of yellow fluorescent protein (YFP) have been described previously (Guirimand et al., 2011; Simkin et al., 2011; Thabet et al., 2011). The coding sequences of these fusion proteins were driven by the constitutive Cauliflower Mosaic Virus CaMV 35S promoter to express high levels of proteins. The "peroxisome"-cyan fluorescent protein (CFP) marker (CFP-SKL, CD3-977) generated by addition of a PTS1 (SKL) at the C-terminus of CFP to allow an efficient peroxisomal targeting (Nelson et al., 2007) has been obtained from the ABRC (http://arabidopsis.org).

Biolistic transformation of *C. roseus* cells and epifluorescence microscopy

Transient transformation of *C. roseus* cells by particle bombardment and fluorescence imaging were performed following the procedures described by Guirimand et al. (2009, 2010). Briefly, *C. roseus* plated cells were bombarded with DNA-coated gold particles (1 μm) and 1100 psi rupture disc at a stopping-screen-to-target distance of 6 cm, using the Bio-Rad PDS1000/He system. Cells were cultivated for 14 h to 38 h prior to being harvested,

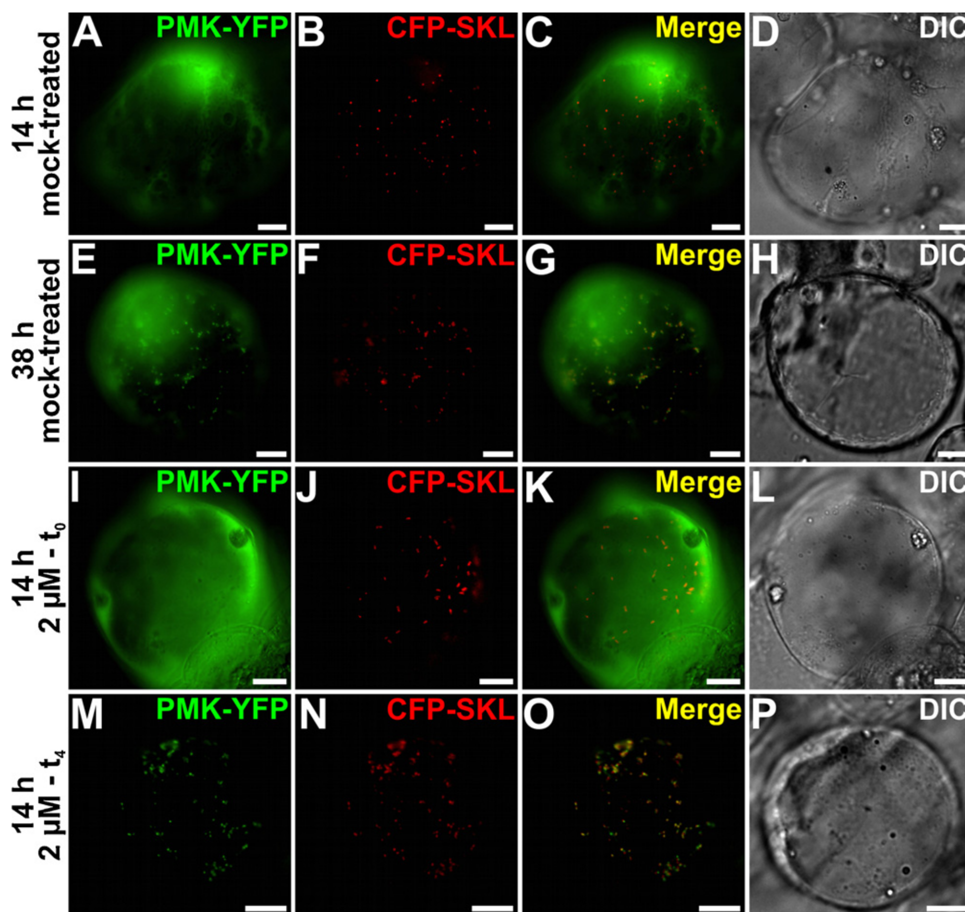

Fig. 1. Effect of CHX treatments on the subcellular localization of the PMK-YFP fusion protein. *C. roseus* cells were transiently co-transformed with plasmids expressing PMK-YFP (A, E, I, M) and CFP-SKL (B, F, J, N) and cultivated 14 h or 38 h prior observation. For the 14-h cultivated cells, a 2 μM CHX treatment or a mock-treatment was performed and cells were observed immediately (t_0) or 4 h (t_4) after CHX treatment. Co-localization of the two fluorescence signals appeared in yellow (C, G, K, O) while merging two individual (green/red) false color images. The morphology (D, H, L, P) is observed with differential interference contrast (DIC). Bars 10 μm.

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