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ARTICLE

Suspension protoplasts as useful experimental tool to study localization of GFP-tagged proteins in *Arabidopsis thaliana*

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ABSTRACT A routinely used protocol is described here including protoplast isolation and PEG-mediated plasmid DNA transformation followed by LSM analysis. We have isolated protoplasts from suspension cultures of wild-type *Arabidopsis thaliana* Col-0, than protoplasts were transformed with different constructs of AtCRK5 gene tagged with GFP. The protoplast isolation and PEG-mediated transformation process took 6-8 hours. Localization studies could be carried out by LSM microscopy in 0-24 hrs followed transformation. Using this method, we could have localized both 35S::cCRK5-GFP and g::gCRK5-GFP fusion proteins in plasma membrane of cell suspension originated protoplasts, while an N-terminal myristoylation site masked version of 35S::cCRK5-GFP and the N-terminal GFP tagged 35S::GFP-cCRK5 fusion proteins could be found in cell nuclei. Mislocalization of the two last fusion proteins is in good agreement with the fact that the N-terminal myristoylation sites of these proteins were impaired. As a conclusion, the Arabidopsis suspension derived protoplast system is very quick tool for identification of protein localization and to provide possibility to obtain preliminarily information on possible protein localization prior to plant regeneration
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KEY WORDS

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PEG mediated transformation
protein localization
fluorescence microscopy

Plant cells missing cell wall are denoted as protoplasts. These cell wall less objects are very useful tools in studying diverse physiological processes, e.g. effects of delivering DNA, RNA and proteins having different origin introduced into protoplasts with several methods such as PEG-mediated fusion, electroporation or microinjection. The transient gene expression system of *Arabidopsis thaliana* mesophyll protoplasts proved to be an versatile and useful system to analyze the function and localization of different proteins involved in certain signalling pathways and cellular processes (Yoo et al. 2007). Since the first report on plant protoplast isolation (Cooking 1960), several papers had been published about the usefulness of protoplast technologies in studying induction of mutations (Cseplo 1994), cell division, cell wall synthesis, embryogenesis, photosynthesis activity, calcium regulation and signalling as well as ion channels regulation via various stresses (Sheen 2001). In our laboratory, we have been using *Arabidopsis thaliana* suspension cultures for various reasons, e.g. for isolation of protoplasts under routine conditions followed by PEG transformation with different cDNA as well as genomic DNA constructs to investigate transient expression of certains genes or localization of their corresponding proteins. AtCRK5 is one of the typical CDPK-related protein kinases belonging to the CRK family (Harmon et al. 2000), which contains all conserved kinase domain, and has N-terminal myristoylation site and C-terminal domain located degenerated EF-hands. Up to now, only one member of this family had partly been characterized, namely CRK3 kinase (Du et al. 2005; Li et al. 2006). In order to get previous information about possible localization and function of our protein kinase, N- and C-terminal GFP tagged fusion proteins were generated via cloning appropriate AtCRK5 cDNA/genomic DNA. Constructs had been introduced by PEG–mediated transformation into A. *thaliana* suspension protoplasts and localization of fusion proteins were monitored in next 24 hrs. We found the same plasma membrane localization pattern using both overexpressing and genomic constructs of C-terminal GFP tagged AtCRK5 protein kinase. Interestingly, nuclear localization of fusion proteins for N-terminal tagged 35S::GFP-cCRK5 and the myristoylation sites masked C-terminal GFP tagged version of 35S::cCRK5-GFP, respectively, had been observed. The reasons for mislocalization of these fusion proteins have been discussed.

Materials and Methods

Suspension culture conditions and maintaince

Suspensions have been maintained and diluted in 1:3 ratios in a weekly period in MS media according to Fulop et al. (2005).

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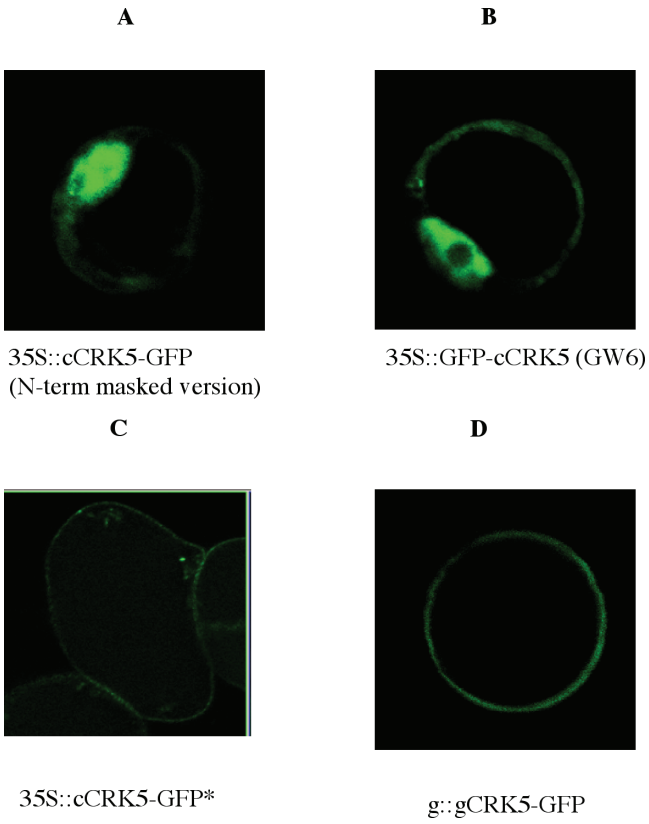

Figure 1. Comparative localization patterns of CRK5 fusion proteins tagged with N and/or C terminal GFP. (A) C-terminal tagged version of 35S::cCRK5 (N-terminal myristoylation site masked version). (B) N-terminal GFP tagged version of 35S::cCRK5. (C) Correct N-terminal site version of 35S::cCRK5 (35S::cCRK5-GFP*). (D) C-terminal tagged version of g::gCRK5.

Protoplast isolation from suspension cultures and PEG mediated transformation

Protoplast were isolated and transformed via PEG treatment as described by Mathur and Koncz (1995).

Plasmid Constructions

1. 35S::GFP-cCRK5 and 35S::cCRK5-GFP constructs

For generating both N and C terminal eGFP gene tagged forms of CRK5 full lenght cDNA, we used Gateway technology system. We used vectors fromhttp://www.psb.ugent.be/gateway/index.php for both N-terminal GFP fusion (35S::GFP-cCRK5, GW6), and C-terminal GFP fusion (35S::cCRK5-GFP). Additionally, a version of 35S::cCRK5-GFP was created by removal of eight extra amino acids obtained under previous cloning procedures resulted in N-terminal masked form of 35S::cCRK5-GFP, and this shortened fragment was cloned into pROK2 plasmid generating the correct N-terminal site for 35S::cCRK5-GFP* fusion protein.

2. g::gCRK5-GFP construct

The CRK5 BAC clone (Arabidopsis Biological Resource Center) was used to create genomic gene fusion which clone contained the genomic promoter region. The translational stop codon of CRK5 was removed, and replaced by eGFP coding region with its stop codon, creating C-terminal in-frame fusion with CRK5 gene (g::gCRK5-GFP).

Fluorescence microscopy

In vivo fluorescent microscopy was applied to study protein localization using Olympus confocal laser scanning microscope (http://www.olympos.com). Localization of fusion proteins had been monitored in 16-18 hours following PEG transformation.

Computer Analysis

Searches for putative protein targeting signals were performed using the TargetP.http://www.cbs.dtu.dk/services/TargetP.

Results and Discussion

Our basic aim was to obtain previous information on possible localization of AtCRK5 protein kinase. Fusion proteins using GFP as a N-terminal and/or C-terminal tag were generated via cloning the appropriate AtCRK5 cDNA/genomic DNA genes. These constructs had transiently been transformed by PEG–mediated transformation into A. *thaliana* suspension protoplasts and localization of fusion products was monitored by confocal laser scanning microscope within 24 hrs. Results are shown in Figure 1. The C-terminal tagged version of 35S::cCRK5-GFP fusion protein in which the N-terminal myristoylation site was probably masked by additional eight aminoacids showed mainly nuclear localization (myristoylation site masked version, Fig. 1A). The N-terminal tagged version of the 35S::GFP-cCRK5 (Fig. 1B) also indicated nuclear localization which is in good agreement with the fact that in this case the CRK5 N-terminal myristoylation site might have also been inactivated by GFP tag. Myristoylation is an irreversible, post-translational protein modification found in fungi, higher eukaryotes and viruses, in which myristic acid is covalently attached via an amide bond to the alpha-amino group of an N-terminal glycine residue. Myristoylation plays a critical role in many cellular pathways, especially in the areas of signal transduction, apoptosis, and extracellular export of proteins (Lu and Hrabak 2002). When a version of 35S::cCRK5-GFP was used from which the extra eight N-terminal aminoacids (probably responsible for arresting myristoylation) were removed (35S::cCRK5-GFP*), fusion protein with correct N-terminal myristoylation site could be found in plasma membranes (Fig. 1C), likewise the C-terminal tagged version of g::gCRK5-GFP (Fig.1D). These results strongly indicate that the CRK5 protein kinase is plasma membrane localized. Finally, the *Arabidopsis* suspen-

sion derived protoplasts combined with PEG-mediated naked DNA transformation is suitable and rapid system to study protein localization prior to plant regeneration.

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