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Comparison of different methods for the isolation of *Arabidopsis thaliana* nuclear membranes

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ABSTRACT: In animal and yeast system, subcellular fractionation has been widely used in studies of protein localization and organelle proteomics. Alternatively, it has not been an effective way of study in plant system because of some experimental limitations. The main aim of this study is to optimize subcellular and subnuclear fractionation of wild-type of *Arabidopsis thaliana* ecotype Colombia (Col-0) leaves by comparing three different methods to isolate nuclear membrane. In the study, we at first optimized nuclear washing steps to remove chloroplast contents from nuclear fractions. By optimizing speed of centrifugation and chemical component of the nuclear washing buffer, purified nuclear fractions was obtained. After measurement of protein amount for each fraction, purity of the fractions was analyzed by western blot assay with some specific cell compartment markers such as anti-Histone3 for nuclear fraction and anti-Rubisco for cytoplasmic fraction. Also, lactate dehydrogenase enzyme assay was used to confirm purity of the fractions. Then, subnuclear fractionation was done to isolate purified nuclear membrane. Three different methods were used to separate the nuclear membrane from whole purified nucleus. Nuclear compartment markers such as anti-Histone3 and anti-Fibrillarin was used in this step. In the study, Inner Nuclear Membrane protein (AtSUN2) was used as a nuclear membrane marker. All things considered, we conclude that the method with DNase digestion and high centrifugation speed (first method) is a more effective way in separation of nuclear membrane fractions because the low centrifugal speed (second method) does not appear to be sufficient for separating the nuclear membrane, and the third method does not seem to be a very effective way as it requires a high centrifugal speed.

Keywords: *Arabidopsis thaliana*, Subcellular fractionation, Nuclear membranes

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INTRODUCTION

The nucleus is known as a distinguished organelle of cell by serving as the control center of cell and home to genetic information in eukaryotic organisms. The role of nucleus in plants has been revealed in the functioning of developmental and adaptive dynamics by supporting increasing new information about the nucleus and its compartments in which has been shown only processing of genetic information so far and is claimed as 'a cell within a cell' by several authors (Rodrigues et al., 2009; Thuleau et al., 2012). The main component of the nucleus comprises nuclear membranes, nucleoplasm, chromatin and nucleolus. Nucleoplasm is encircled by a double-membrane system that has a variety of functions such as organization and the role of the nuclear genome separating nucleus and cytoplasmic environment via around 100 different proteins estimated to localize to these membrane systems (Meier and Brkljacic, 2009) and nucleocytoplasmic transport by regulating exchanges of molecules between these two compartments (Terry et al., 2007; Carmody and Wente, 2009).

In the nuclear envelope, the periplasmic (perinuclear) space separates the outer nuclear membrane (ONM), which continues with the endoplasmic reticulum (ER); but, do not share similar protein composition with ER (Hetzer et al., 2005) and inner nuclear membrane (INM). Nuclear pore complexes (NPCs) that make a connection with ONM and specific protein composition that plays important role in some crucial functions. For instance, some human genetic diseases have been associated with INM proteins such as Lamin B Receptor (LBR), Lamina-Associated Polypeptide1 (LAP1), the LEM (for LAP2, Emerin, MAN1) domain protein family in animal system (Ellis 2006; Worman and Bonne 2007; Wheeler and Ellis 2008).

On the other hand, a small number of INM proteins of animal share homology with plants INM proteins (Boruc et al., 2012) and plants do not have some known animal nuclear membrane proteins; for example, some certain animal INM proteins which can be used as a nuclear membrane marker such as the lamin B receptor (LBR) and Lap/Emerin/Man1 (LEM) domain proteins do not have homologs in plants (Mans et al., 2004; Meier, 2007).

Klarsicht, ANC-1, and Syne Homology (KASH) and Sad1/Unc84 (SUN) proteins create protein bridges by interacting in the perinuclear space in animal systems. These bridges have some functions like making a connection between the nucleus and cytoplasmic cytoskeleton, the regulation of apoptosis and some human diseases such as laminopathies (Burke and Roux, 2009). SUN domain proteins identified in *Arabidopsis* as AtSUN1 and AtSUN2, which are conserved and homologs between animal and yeast systems, were determined as markers for NE dynamics (Boruc et al., 2012), are located at the NE in *Arabidopsis* plants (Oda and Fukuda, 2011).

Subcellular fractionation methods have been widely known and used to analyze protein localization and function in addition to organelle proteomics in the animal and yeast system for a long time. It bases principally on separation organelles according to their size, shape and density via differential and gradient centrifugation techniques. Additionally, it has become an effective tool with accumulation of biochemical knowledge in studies of plant proteomics. Subcellular fractionation generally includes three main steps; homogenization to disrupt cell and separate organelles without any damage under certain conditions and with specific homogenization medium, centrifugation and specific marker assays to measure the level of purity of fractions as a final step.

Currently, scientists are focusing on studies in understanding individual genes functions via plant lines with gene knockouts and over expression methods, but they must deal

with the characterization of phenotypic changes where occurred in plant system by the way of documentation of protein localization, abundance and functions to introduce these transgenic lines as commercial products (Haynes and Roberts, 2007).

In addition to subcellular fractionation, there are some methods to determine the subcellular localization of proteins such as bioinformatic tools and epitope tagging and microscopy. The most widely accepted reporter proteins in the subcellular localization analysis are fusion proteins including green fluorescent protein (GFP) despite some limitations; for instance, protein may be forced a to take place in non-physiological location due to use of non-physiological promoters to express the tagged protein (Piedras et al., 2000).

The objective of this study is to find an efficient method in getting purified nuclear membrane fractions by comparing three different existent methods.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Wild-type of *Arabidopsis thaliana* ecotype Colombia (Col-0) was used in all experiments. *Arabidopsis* seeds were surface sterilized using bleach. Briefly, the seeds were incubated with 70% ethanol in a microcentrifuge tube by shaking for 2 minutes. Then, they were centrifuged at 10 000 g for 30 seconds to remove ethanol. Second incubation was done with 100% (v/v) commercial bleach by shaking for 5 minutes and spun at 10 000 g for 15 seconds. After removing bleach and finishing surface sterilization, seeds were washed with sterile double distilled H_2O at least 6 times by spinning. Then, the seeds were germinated on MS medium (Sigma-Aldrich, St. Louis, MO, USA) with 1% sucrose (pH 5.7 and 0.8% agar) in petri plates (100 mm × 15 mm) and kept in darkness at 4°C for 2 days to break dormancy. After plate were germinated into the growth chamber for one week, they were transferred to soil pot into the same growth chamber with a 16 hours photoperiod at 23/23°C (day/night). We collected leaves from three-week-old plants for subcellular fractionation assays.

Subcellular Fractionation for Nucleus and Cytoplasm

Firstly, we used 3-week-old *Arabidopsis* plants to obtain purified nuclear and cytoplasmic fractions by modifying previously described protocol (Figure 1) (Cheng et al. 2009). Leaf materials (2 g) from soil grown plants were ground in liquid nitrogen and mixed with 4 mL lysis buffer (20 mM Tris-HCl pH 7.4, 25% glycerol, 20 mM KCl, 2 mM EDTA, 2.5 mM MgCl2, 250 mM sucrose, 10 mM 2-mercaptoethanol,1 mM PMSF). Homogenate was filtered through 4 and 8-layer cheesecloth and centrifuged at 1 500 g and at 4 °C for 10 minutes to pellet the nuclei. Supernatant was separated from pellet (P1 fraction) as S1 fraction into new eppendorf tubes. The supernatant (S1 fraction) was re-centrifuged at 13 000 g and 4 °C for 15 minutes, and supernatant of this fraction (S2) was collected as the cytoplasmic fraction and pellet (P2 fraction was discarded). P1 fraction (the nuclear pellet) was carefully suspended in nuclear washing buffer (20 mM Tris-HCl, 25% glycerol, 2.5 mM MgCl2, 0.1% Triton X-100, 10 mM 2-mercaptoethanol, 1 mM PMSF) and centrifuged at 1.500 g for 10 minutes at 4 °C. This step was repeated for 6-7 times until no green color can be seen. Each nuclear fraction was suspended into 0.5 mL nuclear wash buffer and kept at -80 °C for several weeks.

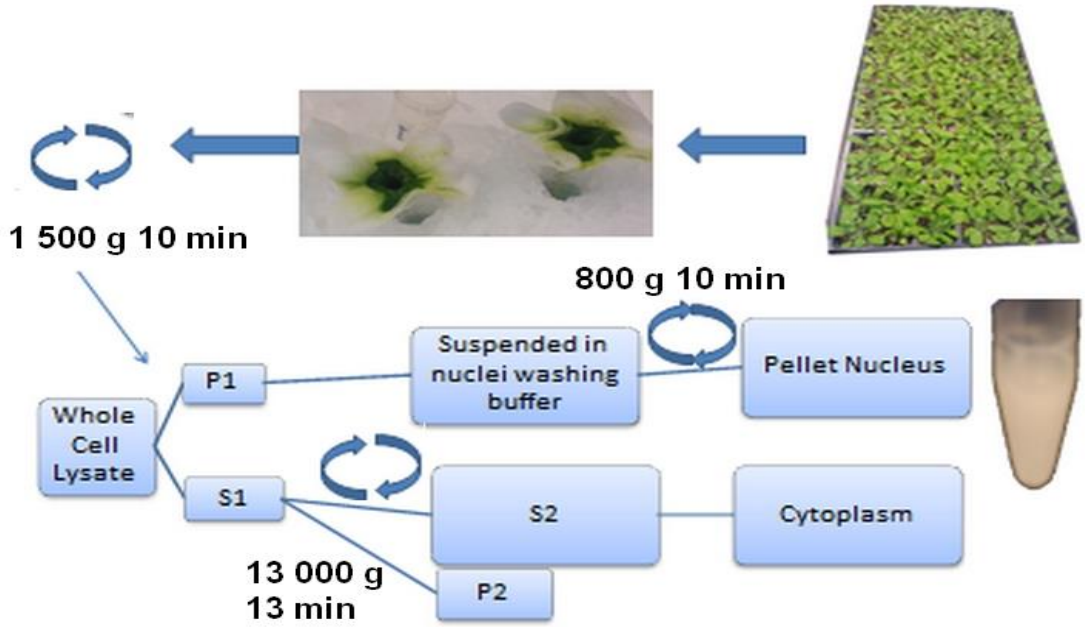

Figure 1. Schematic diagram of subcellular fractionation procedure

Subnuclear Fractionation

As a second step of our research, we did attempt to select appropriate methods for subnuclear fractionation and optimize the best method for *Arabidopsis* plant leaves. Thus, we choose three different existent methods for this assay.

Method 1

The protocol is modified from Kay et al. (1972). Firstly, the nuclei were suspended with nuclei suspension buffer (0.25 M sucrose, 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$) in centrifuge tubes (Beckman Coulter, IN, USA) with 0.1 mM $MgCl_2$ to a concentration of 3 mg protein per mL by gently vortexing to mix suspension well. After 5 µg mL^{-1} DNase was added to final concentration, the suspension was completed with 4 volume sucrose buffer (10 mM Tris-HC1, 0.1 mM $MgCl_2$, 5 mM 2-mercaptoethanol in sucrose pH 8.5). Following incubation of this mix at room temperature for 20 minutes, digestion of nuclei was terminated with an equal volume of ice-cold distilled water and centrifuged for 15 minutes at 36 000 g. Second digestion was applied to get pellet of the first centrifugation. The pellet was suspended in sucrose buffer (pH 7.4) with 1 µg mL^{-1} DNase to final concentration and incubated 25 minutes at room temperature until termination with an equal volume of distilled water. Next, the suspension was centrifuged 15 minutes at 36 000 g. Final pellet was collected as nuclear membrane fraction at the end of the centrifugation process, suspended into nuclear washing buffer and kept on -80 °C for several weeks.

Method 2

The protocol was adapted from Matunis (2006) with some modifications. Nuclear pellet from purified nucleus was suspended in 1 mL of lysis buffer (0.1 mM $MgCl_2$, 1 mM PMSF, 5 µg mL^{-1} DNase, 5 µg mL^{-1} RNase) by adding drop-wise and vortexing at room temperature. Then, suspension was transferred to a Beckman centrifuge tube and completed with 4 volume extraction buffer pH 8.5 (10% sucrose w/v, 20 mM triethanolamine with pH 8.5, 0.1 mM $MgCl_2$, 1mM PMSF). Incubation was done at room temperature for 15 minutes. Total suspension was underlaid with an equal volume of ice-cold sucrose cushion solution (30%

sucrose w/v, 20 mM triethanolamine with pH 7.5, 0.1 mM $MgCl_2$, 1 mM PMSF). Next, nuclear envelope was obtained as pellet by centrifugation at 4 000 g for 15 minutes and suspended with nuclear washing buffer and kept at -80 °C for several weeks.

Method 3

This protocol is described previously by Philipp et al. (1976). The nuclear pellet was suspended in 0.1 mM Tris-HCl (pH 7.5). Then, sonication was performed 10 times with 3 second cooling intervals (QSonica Sonicator, Q55). The suspension was centrifuged at 150 g for 5 minutes. The supernatant was layered directly on top of a 66% (w/v) sucrose solution and centrifuged at 75 000 g for 90 minutes in ultracentrifuge. Later, the pellet was recovered as nuclear membrane fraction and suspended with nuclear washing buffer and kept at -80 °C for several weeks.

Quantification of Protein Concentration

We used protein assay kit (Bio-Rad, USA) to quantify protein concentration. The assay was done according to manufacturer protocol. Protein concentration of samples was determined after preparing standard curve of 595 nm absorbance versus micrograms protein by reading via microplate reader (Bio-Tek , Synergy HT, Winooski, VT, USA).

Lactate Dehydrogenase Assay (LDH)

Firstly, a standard curve was prepared for NADH dilutions by reading absorbance at 340 nm in microplate reader (Bio-Tek, Synergy HT, Winooski, VT, USA). Then, 5 µL of the sample was added to 200 µL of pre-incubated reaction medium at 30 °C for 20 minutes including 100 mM Tris-HCl buffer, 10 mM of pyruvate and 0.3 mM NADH and the absorbance were read at 340 nm per minute. Decreasing in absorbance coming from the oxidation of NADH is determined as the reaction velocity with reference to the general equation of catalytic function of LDH. One unit is defined as the oxidation of one micromole of NADH per minute at 25 °C and pH 7.3.

Protein Separation with SDS-Polyacrylamide Gel Electrophoresis

Once the cast was assembled and tested for leaks, polyacrylamide gel concentration was prepared according to protein size because pore size will affect protein migration through the gel. Consequently, we set 8, 10 and 12 % separating gel concentration for our experiment. Firstly, separating solution was directly transferred into the cast via pipette and overlaid with ddH_2O for 15 minutes to stop air interfering with the crosslinking reaction. After removing the ddH_2O by using blotting paper, the stacking gel (4 %) was directly overlaid on the separating gel, followed by insertion of the comb. Then, gel assembly was put into the gel tank and 1X ris-glycine SDS-PAGE running buffer (Bio-Rad, Hercules, CA, USA) was poured into the tank. 25 µg of the lysates and 10 µL ColorPlus prestained protein marker, Broad Range (7-175 kDa) (P7709S New England Biolabs, UK) were loaded into wells using gel loading tips and ran at 40 mA till bromophenol dye reaches the bottom of the gel.

Transferring Proteins and Developing Western Blot

Transfer of protein from an SDS-polyacrylamide gel onto PVDF membrane (Bio-Rad, USA) was made using the correct size of the gel. Firstly, the membrane prepared for transfer by soaking in methanol. Sponges, blotting paper and the gel removed carefully from the glass plates were soaked in cold transfer buffer for 15 minutes. Then, all of them were sandwiched in a transfer cassette and placed into the gel tank with an ice pack added. Next, 1 X Tris/Glycine transfer buffer (Bio-Rad, USA) buffer was added and the transfer was performed at 300 mA for 1 hour.

Western blotting of the membrane was performed after protein transfer. First, the membrane was blocked with blocking solution (2% skim milk and 0.5% Tween 20) in TBS buffer (10 mM Tris-HCl, pH 7.3, 100 mM NaCl) for 30 minutes on a rocker at room temperature. Following this, the primary antibody shown in the Table 1 at different dilutions according to the specificity of antibody was incubated with the membrane (overnight 4 Cº on the shaker). Three washes of 5 minutes were then made with TBST buffer (TBS buffer and 0.1 % Tween 20) before the addition of the secondary antibody (anti-Rabbit or anti-mouse) at a dilution of 1:2000 for 1 hour. Before developing the blot, it was washed with two changes of TBST and two washes of 5 minutes with TBS buffer. In a separate tube, we mixed black and white ECL solutions in a 1:1 ratio. Then, we aliquoted the solution onto the membrane and waited for 5 minutes. After draining the ECL, we wrapped membrane in plastic and exposed to film. Development of film was made by using with appropriate solutions into the dark room.

Table 1. Primary antibodies used in this study

Antibody	Application/Dilution	Source
Anti-H3	WB/1:2000	BioLegend, San Diego, CA, USA
Anti-RBCL	WB/1:2000	Agriseria, Vännäs, SWEDEN
Anti-SUN2	WB/1:250	Pacific Immunology, Ramona, CA, USA
Anti-FBL	WB/1:2000	Antibodies-online, Atlanta, GA, USA

RESULTS AND DISCUSSION

Nuclear Fractions

In the first experiment, all samples with equal volume were run with Anti-H3 antibody. This antibody has been widely used as nuclear marker in animal and plant systems. We observed that there is no contamination into cytoplasmic fractions. But, quantification of protein amount of each sample may give us to perform the blotting process with equal amount of proteins. That's why we used Bio-Rad protein assay to measure protein amount for each sample. After running equal amount protein, it has been re-demonstrated that there is no signal in cytoplasmic fractions for Histon3 (Figure 2).

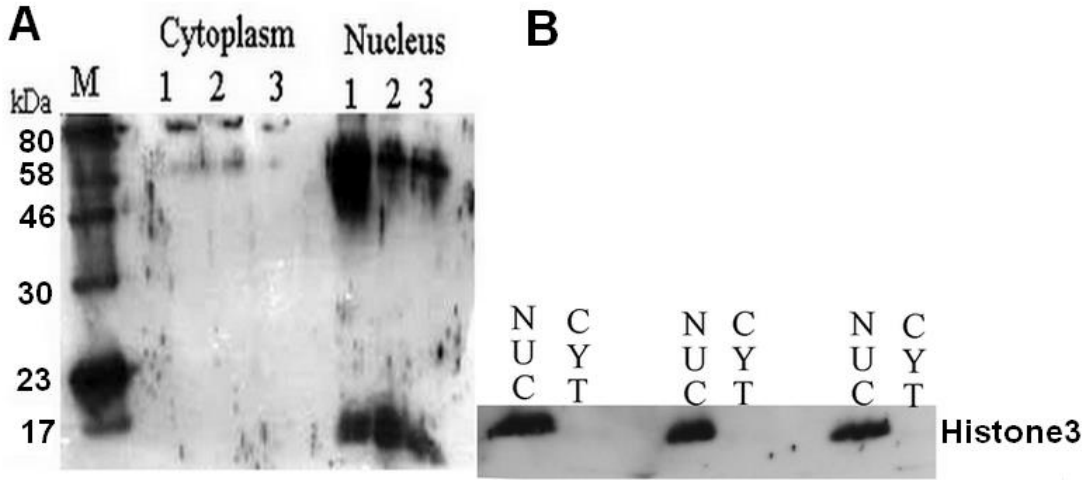

Figure 2. Western blot with anti-Histone 3 nuclear marker with equal amount of protein (25 µg) was loaded for each sample. M (molecular mass markers), NUC (nucleus), CYT (cytoplasm) A. First run B. Second run

Cytoplasmic Fractions

Lactate dehydrogenase (EC 1.1.1.27, LDH) is a soluble enzyme and localized in the cytosol (cytoplasm), which plays a role in the last step in glycolysis as a catalyzer. Consequently, LDH can be used as a quantitative marker enzyme for the intact cell (Renner et al., 2003). Lactate dehydrogenase activity (cytoplasmic marker) protocol was measured by using a standard protocol (Kuznetsov and Gnaiger, 2010). Firstly, we made the standard curve with NADH to calculate the activity of LDH. The enzyme activity was measured as nmol substrate hydrolysed per µg protein in both cytoplasmic and nuclear fractions. Lactate dehydrogenase activity was lower in nuclear fractions samples. This indicates that there is low-level contamination of cytoplasmic fractions into the nuclear fractions (Figure 3).

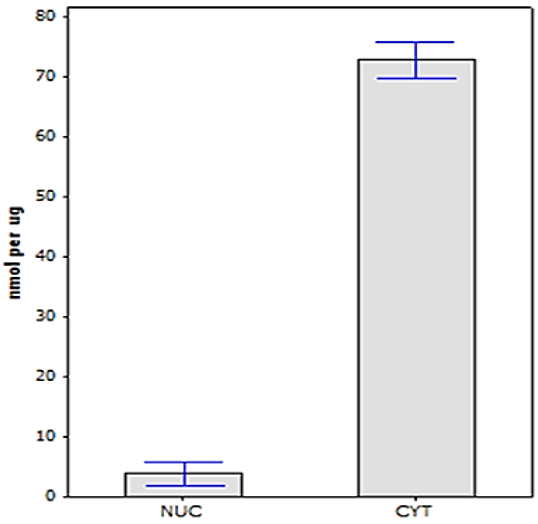

Figure 3. Certain activity of LDH into cytoplasm and nucleus. Two technical and three biological replicate NUC (nucleus), CYT (cytoplasm)

Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase) is known that it plays a role as catalyzer into the rate-limiting step of CO_2 fixation for photosynthetic organisms. The large subunit is coded by the chloroplast rbcL gene, and the small subunit is coded by a family of nuclear rbcS genes in plants and green algae. For *Arabidopsis*, the large subunit of Rubisco is expected to have 52.7 kDa molecular weight.

We made a second confirmation to show the level of purity of nuclear fractions. We used Anti-Rbcl to identify if nuclear fractions have any contamination with cytoplasmic fractions. It was not observed any signals for this protein into nuclear fractions (Figure 4). These results are parallel to Lactate Dehydrogenase assay results.

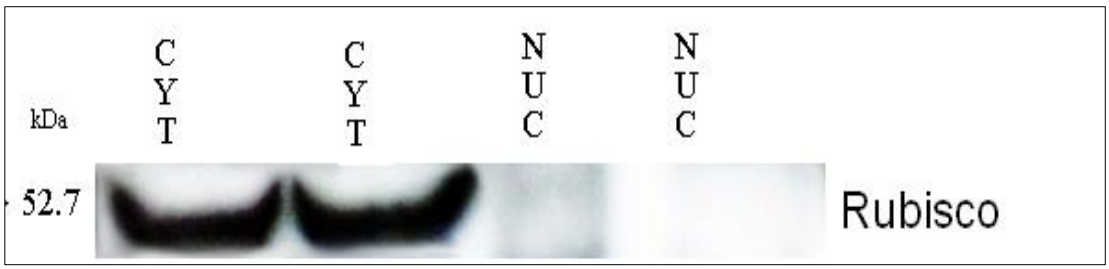

Figure 4. Western Blot with Anti-Rbcl as cytoplasmic marker. NUC (nucleus), CYT (cytoplasm)

Nuclear membrane fractions

We separated nuclear membrane with first method which was mentioned above. After measurement protein amount for each sample, the western blot assay was done with anti-Histone 3. Although the intensity of Histone3 protein was decreased into nuclear membrane fractions, it is expected that there should not be any Histone3 proteins into nuclear membrane fractions.

In the second step, we separated nuclear membrane fractions via three different methods and run three different marker proteins; Histone3, Fibrillarin and AtSUN2. Histone3 was used to determine chromatin contamination into nuclear membrane fractions (Figure 5). Any Histone3 protein into nuclear membrane fractions was not found for the first method. In the second method, we observed Histone3 protein into NMB. Also, there was no signal of Histone3 detected into NMB for third method.

We used Fibrillarin antibody (FBL) as a nucleolar marker which has 34 kDa molecular weight (Figure 6). It was only appeared into nuclear fractions. It has been noticed into neither cytoplasmic fractions nor nuclear membrane fraction for all three methods.

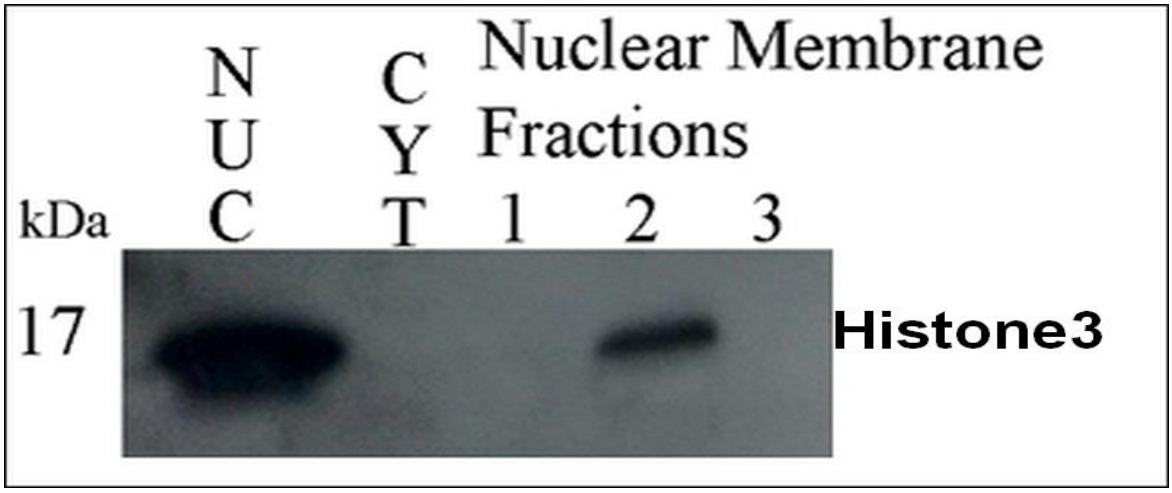

Figure 5. Histone 3 as a marker for nucleoplasm including chromatin. Western Blot with nuclear membrane fractions of three methods. NUC (nucleus), CYT (cytoplasm), Nuclear membrane fractions 1(first method), 2(second method), 3 (third method)

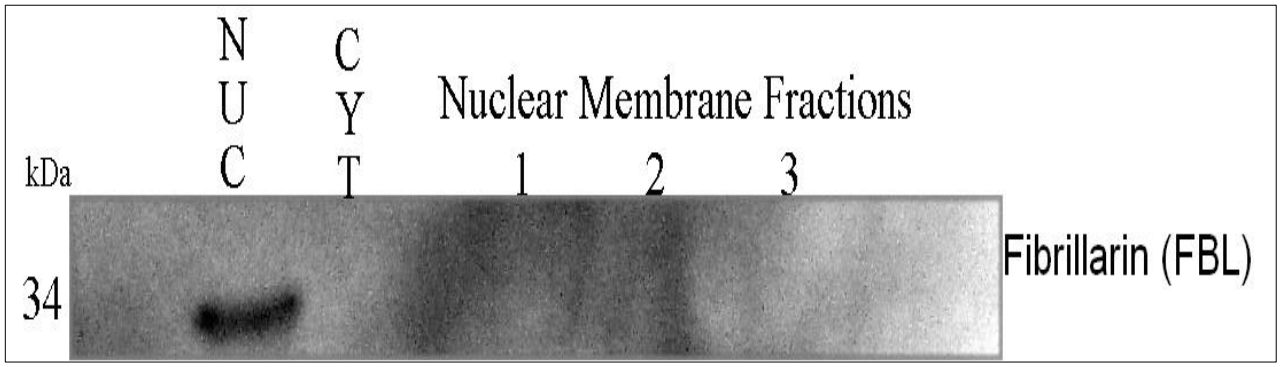

Figure 6. Fibrillarin as a nucleolar marker. Western Blot with nuclear membrane fractions of three methods. NUC (nucleus), CYT (cytoplasm), Nuclear membrane fractions 1(first method), 2(second method), 3 (third method)

AtSUN2 as a Nuclear Membrane Marker

Once we confirmed there is no contamination with both chromatin and nucleolus into nuclear membrane fractions for first and second methods, we determined AtSUN2 protein with 50 kDa molecular weight as a nuclear membrane marker. After the selection of peptide sequence using bioinformatics tools, purified antibody was produced by the Pacific Immunology Company. We run western blot assay with nuclear membrane fractions against anti-AtSUN2. The results indicate that it has been seen into all three nuclear membrane fractions (Figure 7).

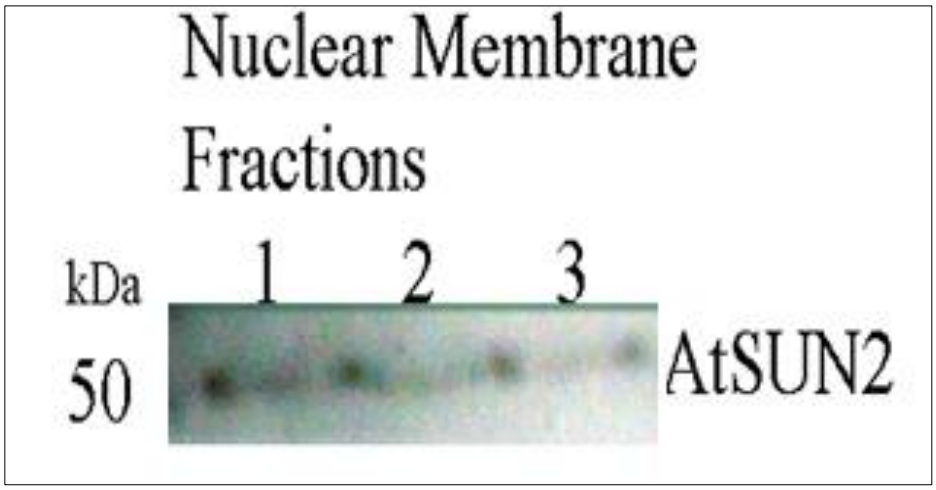

Figure 7. AtSUN2 as a nuclear membrane marker. Western Blot with nuclear membrane fractions of three methods. Nuclear membrane fractions 1(first method), 2(second method), 3 (third method)

As reported in previous studies, we used LDH, which is a glycolytic enzyme in the cytosol, as a cytoplasmic marker in this study (Slabas et al., 2004). When we looked at the LDH activity, we observed a minimal activity in the nucleus fraction, and the presence of Histon3 only in the nuclear fraction showed that we obtained a cytoplasmic and nuclear fraction without too much contamination. A thorough proteomic study of the *Arabidopsis* nuclear matrix was performed previously, and the fibrillarin as indicated herein are shown as nucleolar proteins (Calikowski et al., 2003). In this study, the absence of any fibrillarin in the nuclear membrane and cytoplasmic fractions shows that the nuclear membranes were obtained in pure form. In a previous study, they investigated the two *Arabidopsis* SUN- domain proteins, AtSUN1 and AtSUN2, which share similar structural features with animal and fungal Sad1/UNC- 84 (SUN)- domain proteins are inner nuclear membrane (INM) proteins (Graumann et al., 2010). In the study, we have shown that SUN protein can be used as a marker for *Arabidopsis* nuclear membrane fractions.

CONCLUSION

We achieved to make subnuclear fractionation to get purified nuclear membrane fractions. We used three different methods with some optimization to separate the nuclear membrane from whole purified nucleus. These three methods were differentiated with some details like first method with digestion of DNase and high centrifugation speed, the second method with digestion of DNase and RNase with low centrifugation speed and third method with disruption via sonication and high centrifugation speed. We used some nuclear compartment markers such as Histone3 and Fibrillarin in this assay. However, it is known that determination of nuclear membrane specific marker in plant

system is not easy because some proteins used as nuclear membrane markers in animal systems such as Lamin proteins do not have homologs into plant nuclear membranes (Brandizzi et al., 2009; Graumann et al., 2010). In the study, we determined and used Inner Nuclear Membrane protein (AtSUN2) as a nuclear membrane marker by collecting related information about recent studies. According these results, it has been demonstrated that the first method with DNase digestion and high centrifugation speed is a more efficient way in separation of nuclear membrane fractions because the third method requires centrifugation at very high speeds and therefore does not seem practical.

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