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Studying protein import into chloroplasts using protoplasts

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Dear Editor,

I would like to submit a manuscript for the protocol collection issue entitled “A simple and efficient method for studying protein import into chloroplasts using protoplasts” by Lee et al.

In this protocol paper, we describe the detailed protocol for preparation of protoplasts and use of protoplasts to study protein import into chloroplasts in Arabidopsis. Protoplasts became a powerful and versatile tool for various studies in protein targeting and trafficking in plants. Protoplasts may represent a system very close to the true in vivo system. Therefore, we think this approach can be very useful in elucidating the protein targeting mechanism in plants.

We hope that this manuscript is suitable for publication in your protocol issue and looking forward to hearing from you soon.

Best wishes,

Inhwan Hwang, Professor

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TITLE:
Studying Protein Import into Chloroplasts using Protoplasts

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KEYWORDS:
Protoplasts, chloroplasts, Arabidopsis, protein import, polyethylene glycol, transient expression

SUMMARY:
Here we describe a protocol to express proteins into protoplasts by using PEG-mediated transformation method. The method provides easy expression of proteins of interest, and efficient investigation of protein localization and the import process for various experimental conditions *in vivo.*

ABSTRACT:
The chloroplast is an essential organelle that is responsible for various cellular processes in plants, such as photosynthesis and the production of many secondary metabolites and lipids. Chloroplasts require a large number of proteins for these various physiological processes. Over 95% of chloroplast proteins are nucleus-encoded and imported into chloroplasts from the cytosol after translation on cytosolic ribosomes. Thus, the proper import or targeting of these nucleus-encoded chloroplast proteins to chloroplasts is essential for the proper functioning of chloroplasts as well as the plant cell. Nucleus-encoded chloroplast proteins contain signal sequences for specific targeting to chloroplasts. Molecular machinery localized to the chloroplast or cytosol recognize these signals and carry out the import process. To investigate the mechanisms of protein import or targeting to chloroplasts *in vivo*, we developed a rapid, efficient protoplast-based method to analyze protein import into chloroplasts of Arabidopsis. In this method, we use protoplasts isolated from leaf tissues of Arabidopsis. Here, we provide a detailed protocol for using protoplasts to investigate the mechanism by which proteins are imported into chloroplasts.

INTRODUCTION:

The chloroplast is one of the most important organelles in plants. One of the main functions of chloroplasts is to carry out photosynthesis[1]. Chloroplasts also carry out many other biochemical reactions for the production of fatty acids, amino acids, nucleotides, and numerous secondary metabolites[1,2]. For all of these reactions, chloroplasts require a large number of different types of proteins. However, the chloroplast genome contains only approximately 100 genes[3,4]. Therefore, chloroplasts must import the majority of their proteins from the cytosol. In fact, most chloroplast proteins were shown to be imported from the cytosol after translation[4-6]. Plant cells require specific mechanisms to import proteins from the cytosol to chloroplasts. However, although these protein import mechanisms have been investigated for the past several decades, we still do not fully understand them at the molecular level. Here, we provide a detailed method for preparing protoplasts and exogenously expressing genes in protoplasts. This method could be valuable for elucidating the molecular mechanisms underlying protein import into chloroplasts in detail.

Protein import can be studied using many different approaches. One of these methods involves the use of an *in vitro* protein import system[7,8]. Using this approach, *in vitro*-translated protein precursors are incubated with purified chloroplasts *in vitro*, and protein import is analyzed by SDS-PAGE followed by western blot analysis. The advantage of this approach is that each step of protein import into chloroplasts can be studied in detail. Thus, this method has been widely used to define the components of the protein import molecular machinery and to dissect sequence information for transit peptides. More recently, another approach involving the use of protoplasts from leaf tissues was developed and it has become widely used to study protein import into chloroplasts[9,10]. The advantage of this approach is that protoplasts provide a cellular environment that is closer to that of intact cells than the *in vitro* system. Thus, the protoplast system allows us to address many additional aspects of this process, such as the associated cytosolic events and how the specificity of targeting signals is determined. Here, we present a detailed protocol for the use of protoplasts to study protein import into chloroplasts.

PROTOCOL:

1. Growth of Arabidopsis Plants

1.1. Prepare 1 L Gamborg B5 (B5) medium by adding 3.2 g of B5 medium including vitamins, 20 g of sucrose, 0.5 g of 2-(N-morpholino) ethane sulfonic acid (MES) to approximately 800 mL of deionized water, and adjust the pH to 5.7 with potassium hydroxide (KOH). Add more deionized water bring the total volume to 1 L. Add 8 g of phytoagar and autoclave for 15 min at 121 °C.

1.2. Allow the medium to cool down to 55 °C and pour 20 – 25 mL of the B5 medium into a Petri dish (9 cm in diameter, 1.5 cm in height) at a clean bench. Dry plates for 2 – 3 days, pack them with clean wrap, and keep them in a refrigerator until use.

1.3. Sterilize *Arabidopsis thaliana* seeds with 1 mL of 70% (v/v) ethanol in a centrifuge tube (e-tube) by continuously shaking for 2 – 3 min. Remove the supernatant, add 1 mL of 1% (v/v) sodium hypochlorite solution, and shake continuously for 2 – 3 min. Prepare 100 – 150 seeds per Petri dish.

1.3.1. Remove the supernatants and wash the seeds with 1 mL of distilled water. Repeat this step 4x. Place the sterilized seeds at 4 °C for 3 days to synchronize seed germination.

1.4. Sow 100 – 150 seeds onto B5 plates using a micropipette and seal the plate with surgical tape.

1.5. Grow plants in a growth room with a 16 h/8 h light/dark cycle at 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity, 70% relative humidity and 20 °C temperature for 2 weeks.

2. Preparation of the Plasmid

Note: High-purity plasmids should be used for transformation; the use of a commercial plasmid purification kit is recommended.

2.1. Purify the plasmid (RbcS-nt:GFP) using a DNA isolation kit. To concentrate DNA, perform ethanol precipitation in a 1.5 mL tube and dissolve the DNA pellet in 100 µL of distilled water. Determine the concentration of the plasmid using a spectrophotometer at 260 nm. Dilute the plasmid to a final concentration of ~ 2 µg/µL. Keep the plasmid at -20 °C until use.

3. Isolation of Protoplasts

3.1. Prepare the following solutions.

3.1.1. Prepare the enzyme solution to contain 0.25% (w/v) macerozyme, 1.0% (w/v) cellulase, 400 mM D-mannitol, 8 mM calcium chloride ($CaCl_2$), 0.1% (w/v) bovine serum albumin (BSA), and 5 mM MES. Adjust the pH to 5.6 with KOH.

3.1.1.1. Filter the enzyme solution using a cellulose acetate filter unit with a 0.45 µm pore size. Aliquot 25 mL of the enzyme solution into 50 mL conical tubes and keep at -20 °C. Thaw the enzyme solution at room temperature (RT) and mix well just before use.

3.1.2. Prepare a 21% (w/v) sucrose solution by dissolving 21 g of sucrose in 100 mL of deionized water and autoclave the solution.

3.1.3. Prepare W5 solution to contain 154 mM sodium chloride (NaCl), 125 mM $CaCl_2$, 5 mM potassium chloride (KCl), 5 mM glucose, and 1.5 mM MES. Adjust the pH to 5.6 with KOH and autoclave the solution.

3.1.4. Prepare MaMg solution to contain 400 mM D-mannitol, 15 mM magnesium chloride ($MgCl_2$), and 5 mM MES. Adjust the pH to 5.6 with KOH and autoclave the solution.

3.1.5. Prepare 1 M D-mannitol and 1 M calcium nitrate stock solutions to make the PEG solution. Autoclave these solutions.

3.2. Harvest intact leaf tissues from 2 weeks-old plants from ~ 1 – 2 B5 plates using a surgical knife and place the harvested leaves into a 50 mL conical tube containing 25 mL of enzyme solution. Place the conical tube containing the enzyme solution and leaf tissues horizontally on a rotary shaker with gentle agitation in the dark. It takes 8 – 12 h for full digestion of leaf tissues.

3.3. At 8 – 12 h after incubation, pour the enzyme solution (containing protoplasts released from leaf tissues) into a fresh Petri dish through a mesh with 140 µm pore size. Carefully layer the protoplast-containing enzyme solution on top of 25 mL of 21% (w/v) sucrose solution and centrifuge it at 98 x g for 10 min with the lowest acceleration and deceleration settings in a swinging-bucket rotor.

3.4. Using a Pasteur pipette, carefully transfer the protoplasts from the upper-most layer (enzyme solution) and at the interface between the enzyme solution and sucrose solution to a 50 mL conical tube containing 25 mL of W5 solution. Centrifuge this tube at 51 x g for 6 min. At this stage, protoplasts will be in the pellet at the bottom of the conical tube.

3.5. Discard the supernatant carefully using a pipette without disturbing the protoplasts pellet. Add 25 mL of W5 solution, and gently resuspend the protoplasts. Incubate the protoplast solution in a 4 °C refrigerator for at least 1 h for stabilization.

4. Protoplast Transformation using Polyethylene Glycol

4.1. Prepare a 40% PEG solution by adding 4 g of PEG 8000, 4 mL of 1 M mannitol solution, 1 mL of 1 M $Ca(NO_3)_2$, and 1.8 mL of distilled water into a 50 mL conical tube and mix well. Dissolve PEG by heating in a microwave oven for 20 – 30 s. Place the 40% PEG solution at RT for cooling down.

Note: The 40% PEG solution should be prepared freshly every time. If protoplasts are not pelleted completely during the 4 °C incubation in the refrigerator, centrifuge the material at 46 x g for 2 min.

4.2. Remove the supernatant carefully but completely and add MaMg solution to the protoplast pellet to yield the concentration of 5 x 10^6/mL.

Note: The number of protoplast can be determined by a hemocytometer viewed under a microscope.

4.3. Place 10 µg of the plasmid DNA each empty 13 mL round-bottom tube and add 300 µL of protoplast solution using a pipette.

Note: The end of the pipette tip should be cut off. Whenever protoplasts are sampled, the protoplast-containing solution should be resuspended right before pipetting so that the same number of protoplasts is added to each tube.

4.4. Mix the plasmid DNA with protoplasts by gently rotating the tubes and immediately add 300 µL of 40% PEG solution using a pipette. Mix gently but completely by rotating tubes and incubate for 30 min at room temperature; tilt the tube almost horizontally and rotate it several times.

4.5. Add 1 mL of W5 solution and mix gently but completely by rotating the tube by hand in a similar manner. Incubate the sample for 10 min at room temperature.

4.6. Repeat this step two times more using 1.5 mL and 2 mL of W5 solution, respectively. Incubate for 30 min after the final addition of W5 solution.

4.7. Centrifuge at 46 x g for 4 min. Discard the supernatant and add 2 mL of W5 solution. Mix gently but completely. Incubate at 22 °C in a dark chamber.

5. Analysis of the Protein Import into Chloroplasts

Note: After PEG-mediated transformation of protoplasts, incubation time ranges from 8 to 24 h.

5.1. Fluorescence Microscopy

5.1.1. Place 10 µL of the protoplast solution on a slide glass using a pipette with a trimmed tip and carefully cover with a coverslip to avoid damaging the protoplasts.

5.1.2. Place the slide on the stage of a fluorescence microscope equipped with excitation/emission filter sets for observing green fluorescent protein (GFP) and chlorophyll auto-fluorescence.

5.1.3. Capture images with a cooled charge-coupled device (CCD) camera and process images using an image-editing software to produce pseudo-color images.

5.2. Total Protein Extraction and Immunoblotting

5.2.1. Prepare denaturation buffer containing 2.5% (w/v) sodium dodecylsulfate (SDS), and 2% (v/v) 2-mercaptoethanol.

Note: Protein import into chloroplasts can be quantified by measuring the degree of transit peptide processing *via* immunoblot analysis using anti-GFP antibody.

5.2.2. Transfer protoplasts into a centrifuge tube and centrifuge at 46 x g for 4 min.

5.2.3. Remove the supernatant and add 80 µL of denaturation buffer. Vigorously vortex for 5 s and add 5x SDS sample buffer (250 mM Tris-Cl (pH 6.8), 0.5 M DTT, 10% (w/v) SDS, 0.05% (w/v) Bromophenol blue, and 50% (v/v) glycerol). Mix well and boil for 10 min.

5.2.4. Subject this protein sample to standard SDS-PAGE and immunoblotting with anti-GFP antibody.

REPRESENTATIVE RESULTS:

The import of proteins into chloroplasts can be examined using two approaches: fluorescence microscopy and immunoblot analysis after SDS-PAGE-mediated separation. Here, we used RbcS-nt:GFP, a fusion construct encoding the 79 N-terminal amino acid residues of RbcS containing the transit peptide fused to GFP. When proteins are imported into chloroplasts, green fluorescence signals from the target protein RbcS-nt:GFP should merge with the red fluorescent signals from chlorophyll auto-fluorescence, as examined by fluorescence microscopy (**Figure 1**). The close overlap of the two signals indicates protein import into chloroplasts. Often the GFP signals are spread throughout the chloroplasts or are concentrated at the center of chloroplasts, with weakly diffuse signals throughout the chloroplasts, depending on the individual protein. The import of proteins can be confirmed by immunoblot analysis using GFP antibody. Total proteins are prepared from protoplasts and separated by SDS-PAGE followed by Western blot analysis (**Figure 2**). In most cases, two protein bands should be observed in the immunoblot if a protein was properly imported into chloroplasts: the upper band corresponds to the full-length precursor and the lower band to the processed form after import into chloroplasts. The amount of the processed form of the protein should increase in a time-dependent manner. Such results would imply that the protein RbcS-nt:GFP is imported into chloroplasts in Arabidopsis protoplasts. Moreover, the ratio of the processed form to the total amount of expressed proteins (the processed form plus precursor) can be used as a measure of import efficiency. If necessary, the chloroplasts can be purified from gently lysed protoplasts, and proteins from the chloroplast fraction can be analyzed by western blotting to further confirm the import of proteins into chloroplasts.

FIGURE LEGENDS:

Figure 1. *In vivo* localization of GFP fused to the RbcS transit peptide to chloroplasts.
Images were taken 18 h after transformation under a fluorescence microscope. Green (**a**), red (**b**), merged (**c**), and bright (**d**) labels indicate GFP image, chlorophyll image, a merged image of the two signals, and bright field image, respectively. Scale bar = 20 µm.

Figure 2. Western blot analysis of RbcS-nt:GFP to investigate protein import into chloroplasts.
Total protein extracts were prepared from protoplasts and separated by 10% (w/v) SDS-PAGE, followed by western blot analysis using anti-GFP antibody.

DISCUSSION:

We provided a detailed protocol for the use of protoplasts of Arabidopsis to study protein import into chloroplasts. This method is powerful for investigating the protein import process. This simple, versatile technique is useful for examining the targeting of the intended cargo proteins to chloroplasts. Using this method, protoplasts are prepared from leaf tissues of Arabidopsis[11,12] which can be obtained from plants at many different growth stages ranging from very early to fully mature leaves. However, care must be taken when growing plants used for protoplast preparation. One should use very healthy plants, as protoplasts prepared from healthy plants can withstand the many steps involved in PEG-mediated transformation. Another important precaution is to use fresh solutions. Slight changes in the concentrations of the solutions can greatly damage the protoplasts, since they are fragile and very sensitive to osmotic pressure.

We have been using protoplasts to study protein import into chloroplasts[9,13,14]. Based on these studies, we were able to dissect the sequence motifs in various transit peptides. In addition, we used protoplasts to identify the targeting signals (the positively charged region flanking the C-terminus of the transmembrane domain) of proteins targeted to the outer envelope membrane of the chloroplast[15]. Similarly, we used protoplasts to investigate protein import into the mitochondria[10]. Again, we were able to identify many critical sequence motifs in the presequences of mitochondrial proteins. In addition, the outer membrane proteins of the mitochondria also contain a positively charged region flanking the transmembrane domain as their targeting signal[15]. These targeting signals share a high degree of similarity in amino acid composition. Indeed, chloroplast proteins were mistargeted to mitochondria during *in vitro* import experiments[16]. However, chloroplast and mitochondrial proteins are specifically imported into their target organelles *in vivo*. Thus, protoplasts can be used to elucidate the mechanisms underlying how targeting specificity is determined between chloroplasts and mitochondria.

Protoplasts represent an ideal system for analyzing the import of proteins into chloroplasts *in vivo*. However, one caveat is that protoplasts may be under strong stress conditions such as wounding stress. Thus, we cannot rule out the possibility that such stress may affect the import process. Thus, in certain cases, the results should be interpreted with caution when protoplasts are used for protein import experiments.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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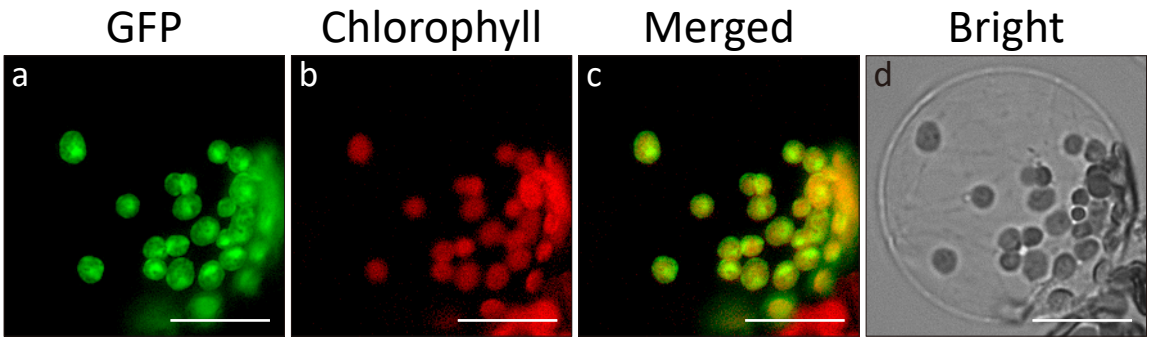

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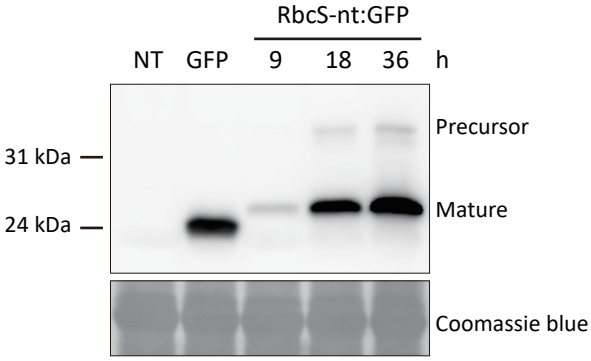

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
GAMBORG B5 MEDIUM INCLUDING VITAMINS	Duchefa Biochemie	G0210.0050
SUCROSE	Duchefa Biochemie	S0809.5000
MES MONOHYDRATE	Duchefa Biochemie	M1503.0250
Agar, powder	JUNSEI	24440S1201
Micropore Surgical tape	3M	1530-0
Surgical blade stainless No.10	FEATHER	Unavailable
Conical Tube, 50ml	SPL LIFE SCIENCES	50050
Macerozyme R-10	YAKULT PHARMACEUTICAL IND.	Unavailable
Cellulase ONOZUKA R-10	YAKULT PHARMACEUTICAL IND.	Unavailable
ALBUMIN, BOVINE (BSA)	VWR	0332-100G
D-Mannitol	SIGMA	M1902-1KG
CALCIUM CHLORIDE, DIHYDRATE	MP BIOMEDICALS	0219463505-5KG
Twister	VISION SCIENTIFIC	VS-96TW
Screen cup for CD-1	SIGMA	S1145
Screens for CD-1	SIGMA	S3895
Petri Dish	SPL LIFE SCIENCES	10090
Pasteur pipette	HILGENBERG	3150102
LABORATORY CENTRIFUGE / BENCH-TOP	VISION SCIENTIFIC	VS-5500N
Sodium chloride	JUNSEI	19015S0350
Potassium chloride	SIGMA	P3911-1KG
D-GLUCOSE, ANHYDROUS	BIO BASIC	GB0219
Potassium Hydroxide	DUKSAN	40
Calcium nitrate tetrahydrate	SIGMA	C2786-500G
Poly(ethylene glycol)	SIGMA	P2139-2KG
Magnesium chloride hexahydrate	SIGMA	M2393-500G
Tube 13ml, 100x16mm, PP	SARSTEDT	55.515
Microscope slides	MARIENFELD	1000412
Microscope Cover Glasses	MARIENFELD	101030
Counting Chamber	MARIENFELD	650030
Axioplan 2 Imaging Microscope	Carl Zeiss	Unavailable
Micro tube 1.5ml	SARSTEDT	72.690.001
2-Mercaptoethanol	SIGMA	M3148-250ML

Sodium Dodecyl Sulfate (SDS), Proteomics Grade	VWR	M107-500G
TRIS, Ultra Pure Grade	VWR	0497-5KG
DTT (DL-Dithiothreitol), Biotechnology Grade	VWR	0281-25G
Bromophenol blue sodium salt ACS	VWR	0312-50G
Glycerol	JUNSEI	27210S0350
Living Colors A.v. Monoclonal Antibody (JL-8)	TAKARA	632381

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Article Title: A simple and efficient method for studying protein import into chloroplasts...

Signature: Inhwan Hwang Date: 05/09/2018

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Reviewer #1:

Manuscript Summary:

In this manuscript, the authors reported a detailed protocol for using protoplasts to study protein import into chloroplasts, which is very helpful for investigating the protein import process in vivo.

The manuscript is suitable for publication in JoVE, and some points I am concerned are listed below.

Major Concerns:

To better adapt to the title "A simple and efficient method for studying protein import into chloroplasts using protoplasts", the protocol of purifying the chloroplasts from protoplasts could be included in this manuscript, which looks like a protocol of how to make protoplasts and gene expression in protoplasts right now.

A : We modified the title.

Minor Concerns:

1. In 1.2, what is the expiration date of the medium plates?

A : Medium plates are usually used up within one month. We recommend to use medium plates within 2 months before they dry out.

2. In 3.1.2, the volume of the deionized water should be pointed, although everybody knows, because 21 g of sucrose has been pointed clearly here.

A : Corrected.

3. In 3.2, some people would like cutting the leaf tissues into very small pieces. Is that required and helpful for the digestion.

A : It would be helpful for the digestion by expanding contact with enzyme solutions but at the same time can have negative effects for the protoplast condition due to more damage and stress

4. In 3.3, the centrifuge time is required.

A : Corrected

5. In 3.4, again, the centrifuge time is required.

A : Corrected

I had a hard time to understand the sentence "carefully transfer protoplasts in the top fraction (enzyme solution) and at the interface between the enzyme solution and sucrose solution to

a 50 mL conical tube ".

A : We modified the expression (the top fraction → the most upper layer)

6. In 4, "Do not invert the tube". Why?

A : The sentence is unnecessary. It is removed

7. In 4.3, please check the dimension of 300 mL.

A : Corrected (300 ml → 300 µl)

Instead of the proper amount of plasmid, could you show the range or an example?

A : Corrected

8. In 4.4, similarly, please check the dimension of 300 mL.

A : Corrected (300 ml → 300 µl)

Reviewer #2:

Manuscript Summary:

This manuscript describes an A-Z protocol for studying chloroplast localization of exogenous proteins using protoplasts, instead of purified chloroplasts. The methods include Arabidopsis growth, protoplasts isolation, infection, and further analysis. An improvement of this method is to use protoplast expression, which is in vivo, instead of in vitro transcription-and-translation and chloroplast importing assay.

Major Concerns:

1. There have been quite some protocols describing protoplast isolation and infection (for examples, Salinas and Sanchez-Serrano, 2006. Methods in Molecular Biology. Vol. 323. Arabidopsis Protocols. Humana Press. and Jarvis, 2011. Methods in Molecular Biology. Vol. 774. Chloroplast Research in Arabidopsis. Humana Press.), therefore the manuscript needs to make it clear how the methods has been improved.

A : We agree with the reviewer on the comment that this is overall the same as those published in previously. However, this manuscript is basis of the video recording. This is to demonstrate exactly how we can do protoplast transformation and how we can analyze the transformed protoplasts using the images and western blotting.

2. To my understanding, previous studies using in vitro translation and importing assay with chloroplasts were mainly to figure out several issues. First, can a protein be imported. Second, will the transit peptide be removed. A critical strategy is to treat the chloroplasts by thermolysin after importing. So that those non-imported proteins could be eliminated. In this way, those chloroplast-associated proteins that only attach to the outer envelope could be distinguished. The method in this protocol is not sufficient to address the same issues. However, I do agree that the method in this manuscript is good for a quick screening of a specific set of chloroplast proteins that are imported and then also processed to remove their transit peptides.

Minor Concerns:

1. The manuscript has some minor typos, such as uL amd mL (e.g. lines 139 and 144).

A : Corrected

2. e-tube should be microfuge tube or something like that (e.g. line 78).

A : Corrected

3. Some characters are not shown properly (e.g. line 85).

A : We made it clear,

4. Line 98, need to make it clear a w/w or w/v ration of enzymes.

A : Corrected

5. Line 112, how much leaves should be used?

A : 2 weeks-old plants of 1~2 B5 plates are usually used.

Reviewer #3:

Manuscript Summary:

This manuscript describes the development of a simple and efficient transient transformation procedure of protoplasts of Arabidopsis to examine the import of proteins into chloroplasts and its suitability for detecting protein-protein interactions using commercially available antibodies. Overall, the manuscript was concise and well written and would make a significant contribution to the research community. However, one major criticism is the lack of some specific technical details in a certain steps of the protocol. These details are critical to researchers who are not familiar with the process and that such information should be present considering the nature of manuscript. Below is a list of specific comments that highlight some of the missing details mentioned above.

Specific comments:

Page 2, line 75 "Before solidification" should read "Allow the medium to cool down to 55 °C,"

A : We corrected as you suggested.

Page 2, line 78 "… 70% ethanol" should be " … 70% (v/v).."

A : Corrected

Similarly, line 79 should also include "(v/v)" after "1% sodium hypochlorite solution"

A : Corrected

Page 3, lines 98, 99 and 103 insert "(w/v)" after "0.25% …, 1.0% …, 0.1% … and 21% …" respectively.

A : Corrected

Page 3, step 3.2, This step should include the number of 2-week old leaves that were used in 25 mL of enzyme solution as the ratio of tissue vs enzyme solution is extremely critical in obtaining a high yield of viable and healthy protoplasts. Also, the authors should put an emphasis here that intact leaves were used here instead of dissected leaf strips were used.

A : We corrected as you suggested.

Page 3, lines 118 and 122 please indicate the length of these centrifugation steps.

A : We added the centrifugation length

Page 3, line 129, should include the concentration of the PEG solution "40%" as the volume use by researchers vary

A : We added 40% for the concentration of the PEG solution

Page 4, lines 138 should indicate the amount of plasmid DNA use per "X" number of protoplasts. Thus, based on the above protoplast concentration, how much DNA would be required for 1.5 million protoplasts. For example, based on the protocol that was optimized in our lab, we found that 5 ug of plasmid DNA/20,000 protoplasts consistently gave the highest transformation rate.

A : No we cannot provide the highest transformation rate. If you increase the amount of DNA the transformation efficiency will increase with the amount of DNA. But at the same time, amount of proteins expressed in individual protoplasts would also increase. Then too much of protein expression in each protoplast may lead to unwanted overexpression-related problems. Therefore, we found that 10 ug of plasmid DNA with the size of around 3-4 kb in $1.5x10^6$ protoplasts appears to be optimal condition for both the transformation efficiency and expression level.

Page 4, line 139, Also, the unit of protoplasts "300 mL" should be corrected to "µL"

A : Corrected

Page 4, line 144 "300 mL" should be corrected to "300 µL"

A : Corrected

Page 4, lines 156 and 159 "Fluorescent …." should be corrected to " Fluorescence ….".

A : Corrected

Page 4, line 170 "80 mL" should be corrected to " 80 µL".

A : Corrected

Page 4, line 171, specify the composition of the 5x SDS PAGE sample buffer.

A : The composition of the solution is described.

Page 7, lines 293-296 reference 15, please change the upper case letters to lower case in the article title.

A : Corrected

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

A : We revised carefully.

2. Please shorten the title. Suggested: studying protein import into chloroplasts using protoplasts

A : We changed the title as you suggested

3. Figure 1: Please label the panels. Please use larger figures with higher resolution.

A : We labeled panels and saved figures of the original size for higher resolution.

4. Figure 2: Please use SI abbreviations for time: h instead of hr.

A : Corrected.

5. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

A : Corrected.

6. Please include a Summary that clearly describes the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

A : We added a Summary

7. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

A : We described protocol steps in more detail.

8. What is an e-tube?

A : It is an abbreviation of the Effendorf-tube. We described it in the manuscript.

9. 2.1: How much is used to dissolve?

A : 100 µl of distilled water

10. 3.3: Centrifuge for how long?

A : It’s 10 min.

11. Please specify all centrifugation time lengths.

A : We specified time lengths.

12. 4.3: What is the proper amount?

A : It’s 10 µg

13. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

A : We highlighted steps

14. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

A : We organized highlighted steps according to the logical flow.

15. Please do not abbreviate journal titles.

A : Corrected