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.