PURIFICATION OF CHLOROPLAST INTERACTION PARTNERS OF MATRIX ATTACHMENT REGION-BINDING FILAMENT-LIKE PROTEIN 1 (MFP1) IN *ARABIDOPSIS THALIANA*

A Thesis
by
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Abstract

PURIFICATION OF CHLOROPLAST INTERACTION PARTNERS OF MATRIX ATTACHMENT REGION-BINDING FILAMENT-LIKE PROTEIN 1 (MFP1) IN *ARABIDOPSIS THALIANA*

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Long coiled-coil proteins are predicted to be involved in photosynthesis, cytokinesis or plant defense mechanisms via protein-protein interactions. Matrix Attachment Region-Binding Filament-like Protein 1 (MFP1) is a plant-specific, long coiled-coil protein initially believed to be localized in the nuclear matrix but later found to be embedded in the thylakoid membrane of the chloroplast. The exact function of MFP1 is unknown and my goal is to purify and analyze chloroplast-localized MFP1 complex by Tandem-Affinity Purification-Mass Spectrometry method. A transgenic plant line was constructed by inserting a MFP1-TAP (Tandem Affinity Purification) construct in mutant (MFP1 knock-out) *Arabidopsis thaliana* plants. The genotype and success of transformation were confirmed via PCR and the transformation efficiency with and without the elicitor acetosyringone was 41.4% and 1.6% respectively. The T_0 seeds from the transformed plants were again replanted to obtain T_1 plants, which were again confirmed by PCR. Following the insertion of MFP1-TAP construct into the plant genome, the expression of the MFP1-TAP fused protein in the transgenic plants was confirmed by SDS-PAGE and western blot using antibodies against MFP1 and TAP.

For both antibodies the blot showed the same band larger than 82 kDa, which is the size of untagged MFP1, indicating the expression of tagged protein. Localization of MFP1 inside the chloroplasts of the transgenic plants was confirmed by isolating chloroplasts followed by purification of MFP1 and detection by SDS-PAGE and western-blot using antibody against MFP1. A transmission electron micrograph study was done to observe the stacking of the thylakoid membrane where the MFP1 is anchored. No difference in stacking was observed between wildtype, knock-out mutant, and transgenic lines, indicating that overexpression of the tagged MFP1 in the knock-out mutant does not disrupt thylakoid membrane structure. I optimized the TAP method by using different extraction buffers with various detergents. Using NP-40 Alternative as the detergent I was able to purify the protein complex. Purification was confirmed by SDS-PAGE and western-blot using antibody against MFP1. However, the concentration of the protein purified was too low to be used for Mass Spectrometry to identify the protein binding partners of MFP1. The protocol I followed must be further modified to purify enough MFP1 complex.

Acknowledgments

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I also would like to thank Alison DeShields for her assistance in optimizing the Tandem Affinity Purification method and allowing me to use the vector she cloned for her own research work. I am also grateful to my parents for their love, faith and emotional support in me.

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Introduction

Coiled-coil domains are protein oligomerization motifs that consist of two or more amphipathic alpha helices wrapped around each other forming a supercoil complex structure (Burkhard et al. 2001). They are characterized by a heptad repeat pattern in the amino acid sequences; the first and fourth positioned amino acids are hydrophobic, and amino acids in the fifth and seventh positions are mainly charged or polar in nature (Mason and Arndt 2004). Hydrophobic side chains are packed in the formation of a hydrophobic core; a structure that provides stability to the coiled-coil domains (Burkhard et al. 2001). Coiled-coil proteins are highly versatile and involved in diverse cellular functions inside the cell due to the variation in the number of heptad repeats and monomers from protein to protein (Rose and Meier 2004).

Coiled-coil proteins are ubiquitously found in most organisms, representing approximately 10% of all proteins in eukaryotes (Burkhard et al. 2001). They are divided into two types based on the presence of short or long coiled-coil domains: 1) short coiled-coil domains have six or seven heptad repeats; referred to as leucine zipper (bZIP), 2) long coiled-coil domains have several hundred amino acids in their coiled-coil domains. The function of these domains differs in a variety of locations in the cell (Venkatakrishnan et al. 2013), and they perform their function by protein-protein interactions (Rose and Meier 2004). Unlike long coiled-coil proteins in plants, long coiled-coil proteins in animals and yeast have been studied well. In addition, identified plant-specific long coiled-coil proteins

are predicted to be involved in processes such as photosynthesis, cytokinesis or plant defense mechanisms via protein-protein interactions, both by homodimerization and heterodimerization of the coiled-coil domains and by interactions with structurally dissimilar proteins. However, the function of many known coiled-coil proteins in plants are still unknown (Gindullis and Meier 1999; Venkatakrishnan et al. 2013).

Matrix attachment region-binding filament-like protein 1 (MFP1) is a plant-specific long coiled-coil protein originally discovered by Meier et al. (1996) in young tomato (*Solanum lycopersicum*) fruit as a protein binding to Matrix Attachment Region (MAR) DNA by south-western screening. Chromatin interacts with the nuclear matrix via MARs which are several hundred base pairs long, AT-rich DNA sequences located in the noncoding regions of the DNA. They help in the higher order organization of chromatin and regulation of gene expression. Two functional domains are present in MFP1: An N-terminal domain of 125 amino acids containing two hydrophobic regions with one showing similarity to transmembrane domains and a C-terminal domain of 258 amino acids, which is part of the α-helical coiled-coil domain and contains the DNA-binding activity. The C-terminal domain has novel structural motifs that might be the *in vivo* target for MAR DNA binding activity of MFP1 (Meier et al. 1996).

Gindullis and Meier in 1999 found that the N-terminal domain of MFP1 was necessary for its localization at the nuclear periphery in structures that are part of isolated nuclei and the nuclear matrix. Likewise, both the hydrophobic domains present in the N-terminal domain are required for the correct localization of MFP1. Among the two

hydrophobic regions, the first transmembrane-like domain might be assisting nuclear membrane attachment of MFP1 (Gindullis 1999).

A study done by Jeong et al. (2003) proved MFP1 is not only present in the nuclear matrix, but also embedded in thylakoid membranes of the chloroplasts, with the majority of MFP1 found in chloroplasts compared to nuclear matrix. Bioinformatics analysis of the amino acid sequence of MFP1 in *Arabidopsis thaliana* (AtMFP1) using ChloroP and TargetP to predict chloroplast targeting peptides in the N-terminal domain, and SignalP to predict signal peptides for association with the thylakoid membrane indicated the import of MFP1 inside the chloroplast and thylakoid membrane. The primary location of MFP1 was studied with tomato MFP1 fused with GFP (LeMFP1-GFP) and a Mito Tracker to differentiate organelles. As a result, MFP1 was localized in the plastid and the nucleus. This result was further confirmed doing immunoblotting using nuclei, nuclear matrix and plastids. Clearly higher concentration of MFP1 was shown inside the plastid than nuclear matrix. Chloroplast fractionation experiments showed MFP1 inside the chloroplast anchored in the thylakoid membrane with its transmembrane domain and with the majority of the protein and C-terminal domain in the stroma (Jeong et al. 2003).

Samaniego et al. (2006b) tested for the dual localization of MFP1 in Arabidopsis, tobacco and tomato using antibodies raised against Arabidopsis, tobacco and tomato MFP1 by confocal immunofluorescence microscopy and immunogold electron microscopy. This was done to confirm that the presence of MFP1 in both locations is a product of a MFP1 gene. Immunofluorescence microscopy was done isolating nuclei from tomato, Arabidopsis and tobacco and using MFP1 antibody. As a result, it showed the general distribution of

MFP1 in all the three species as listed before. Following this, immunolabeling was done by isolating the nuclear matrix and using MFP1 antibody. Similar results were obtained but there was variation in the expression level which might be due to the multiprotein complex formed in each species. Thus, an electron micrograph of leaf nuclei was done which showed variation in the size and distribution of heterochromatin patches in the three species. To compare MFP1 in nuclei and the chloroplast in Arabidopsis, tomato and tobacco, the nuclei and chloroplasts were co-purified and tested against two antisera which confirmed the MFP1 signal in both compartments. Finally, to confirm that both nuclear and chloroplast localized MFP1 is expressed by the same gene, wildtype and T-DNA inserted (MFP1 knockout) lines were used. Wildtype showed the presence of MFP1 which was absent in T-DNA inserted lines. Therefore, this paper confirms the dual location of MFP1 in nucleus and chloroplasts expressed by a single gene (Samaniego et al. 2006b).

In eukaryotic cells, DNA is also present in plastids and mitochondria in addition to the nucleus. DNA present inside bacteria, plastids and mitochondria is organized similar; DNA is bound with proteins and called nucleoids. Chloroplast DNA binding activity of AtMFP1 was tested by using wildtype Arabidopsis and T-DNA inserted (MFP1 knockout) Arabidopsis plants. MFP1 was expressed only in the wildtype and absent in the inserted line, which corresponded to the DNA-binding activity only in the wildtype and not in inserted lines (Jeong et al. 2003). The DNA-binding activity of MFP1 in plastids and nuclear matrix is reduced by phosphorylation by protein kinase CKII (Jeong et al. 2004; Samaniego et al. 2006a).

The thylakoid membranes of higher-plant chloroplasts exhibit a stacked structure very similar in appearance to that of the Golgi apparatus. Due to the similarities in the protein structure of MFP1 and golgins, which are involved in golgi membrane stacking, and the similarities in structure between the Golgi apparatus and the thylakoid membrane, it was hypothesized that MFP1 may be involved in thylakoid membrane stacking. Havighorst (2012) studied the role of MFP1 in stacking the thylakoid grana but no significant difference was seen in the grana stacks between mutant and wild type Arabidopsis plants. If MFP1 was associated with the grana layer stacking, the knock-out mutant would show fewer membranes per grana stack.

Because coiled-coil domains facilitate protein-protein interaction, another hypothesis is that MFP1 functions through protein-protein interaction. A novel plant protein MFP1 attachment factor 1 (MAF1) interacting with MFP1 was identified by Gindullis et al. in 1999 through yeast two-hybrid screening. MAF1 is a small (16.2 kDa) hydrophilic protein rich in serine/threonine residues well conserved among higher plants. Yeast two-hybrid and *in vitro* assays confirmed the interaction between MAF1 and MFP1. Subcellular localization of MAF1 at the nuclear envelope was shown by expressing a sandwich fusion MAF1-mGFP-MAF1 construct to have a fusion protein large enough (59 kDa) to avoid passive diffusion through the nuclear pores. GFP fluorescence clearly accumulated at the nuclear envelope, confirming the location. Overlapping localization of MAF1 and MFP1 at the nuclear envelope supports that they are parts of the same macromolecular complex and interact *in vivo* at the nuclear envelope (Gindullis et al. 1999). However, no MAF1 signal was detected in chloroplasts and the plastidic interaction partners of MFP1 are still unknown.

A study done by Havighorst (2012) predicted MFP1 is involved in protein complex formation and homeostasis in chloroplast. The MFP1 protein was detected in wildtype samples in immunoblots of 1D Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) gels. Bands were detected in the range of 160 kDa, 400 kDa and > 880 kDa. No signal was detected between the lower molecular weight complexes (~ 150-500 kDa) and these higher molecular weight complexes (> 880 kDa), indicating MFP1 is a member of multiple distinct protein complexes inside the chloroplast. The presence of MFP1 in several high molecular mass complexes is also corroborated by chloroplast membrane proteomics studies in maize, which detected MFP1 homologs in high molecular mass complexes of ~250 kDa, ~500 kDa, and ~700 kDa (Majeran et al. 2008). These MFP1-containing complexes likely represent thylakoid-associated complexes, based on the known integral thylakoid membrane localization of MFP1 and a clear lack of MFP1 observed in stroma protein complexes (Jeong et al. 2003; Friso et al. 2004; Olinares et al. 2010).

Full-length Arabidopsis MFP1 has a predicted size of 82 kDa, and processed, mature plastidic MFP1 with the targeting peptides cleaved off is predicted to be 72 kDa in size. However, no signal was detected below 150-160 kDa, suggesting that MFP1 does not exist as a monomer in the chloroplast, but is always found within a complex or as a homodimer (Havighorst 2012). Dimerization of MFP1 has also been previously reported from SDS-PAGE results, which indicated the presence of MFP1 homodimer in onion nuclear matrix fractions (Samaniego et al. 2008). The 2D BN/SDS-PAGE showed a variety of partial MFP1 sizes around 50-55 kDa and smaller in addition to the mature protein, which may indicate alternative splice forms, modification, or cleavage of MFP1 within the complex it is forming. The MFP1 signal detected in higher molecular mass complexes above the 880 kDa marker

size is markedly concentrated in a strong band in the megadalton range (Havighorst 2012). This signal may represent the Arabidopsis transcription complex (1500-1700 kDa, Behrens et al. 2013), based on MFP1's known association with the nucleoid (Jeong et al. 2003).

Due to MFP1's location in the thylakoid membrane, Havighorst tested if loss of MFP1 caused changes in photosynthetic complexes of the thylakoid membrane. No difference was detected between mutant and wildtype plants grown under standard laboratory conditions in either photosynthesis rates or chlorophyll content. However, changes in the relative abundance of several complexes in the MFP1 knock-out mutant compared to wildtype suggested that MFP1 may be indirectly involved in the formation or maintenance of chloroplast protein complexes. To identify the nature of these protein complexes, the apparent size and relative positions of the bands were compared with previously published BN-PAGE data obtained after solubilization of chloroplasts or thylakoids with digitonin (Heinemeyer et al. 2004; Järvi et al. 2011). The results suggested lack of MFP1 reduced the relative abundance of complexes in the size range for PSII monomers, LHCII assembly, cytochrome b_6f and F_1 ATP synthase. On the other hand, a band in the range of PSI-LHCII complexes or PSII super complexes appeared stronger in the mutant. PSI-LHCII complexes were only observed after digitonin solubilization and not with other detergents (Järvi et al. 2011). Since the abundance of complexes appeared to change between wildtype and mutant, MFP1 might facilitate some aspect of the dynamics of the system by affecting protein complex formation or stability.

However, the exact function of MFP1 is unknown. My goal is to identify the function of MFP1 by studying its chloroplast-localized interacting protein partners. My goal is based

on the hypothesis that because coiled-coil domains function as protein-protein interaction motif, MFP1 also should be involved in protein complex formation to perform a function inside the chloroplast.

Various methodologies are available for detecting protein-protein interactions (PPIs) which are based on interaction dynamics, environmental conditions, expression control, reporter output, controls, binary or co-complex protein (Xing et al. 2016). The preferential methods especially used in plant science for PPIs study are: yeast two-hybrid (Y2H), and tandem affinity purification-mass spectroscopy (TAP-MS). Previous attempts to find interaction partners of MFP1 through Y2H, were not able to identify the chloroplast proteins because in classical Y2H system (heterologous system) all fusion protein with activation/binding domains contain nuclear localization signals to import into the nucleus to activate the reporter gene. However, proteins carrying signals to sort into another compartment of the cells like chloroplasts or containing hydrophobic domains (integral membrane protein) may be localized in a different subcellular compartment of the plant cell resulting in possible false positive interactions that are not relevant for plant cell function (Causier and Davies 2002). MAF1, the protein identified as interaction partner of MFP1, was shown to be localized to the nuclear envelope and not the chloroplasts (Gindullis et al. 1999).

To identify chloroplast-localized interacting protein partners of MFP1, I used the TAP-MS method, which is an efficient system for identifying *in vivo* protein interaction partners (Xu et al. 2010). It helps in purification of the protein complex under native physiological condition without knowing the function and structure (Xu et al. 2010). This method is based on a TAP tag fused to either N- or C- terminus of the protein of interest for

two-step purification of protein complex to be analyzed by Mass Spectrometry (Puig et al. 2001). The TAP tag used in the original TAP-MS method consisted of tandem protein A domains that bind to IgG-agarose beads, a calmodulin-binding protein (CBP) domain that binds to calmodulin agarose beads in a calcium dependent manner, and a tobacco etch virus (TEV) protease cleavage site between protein A and CBP cleaved by a TEV protease (Puig et al. 2001; illustrated in Fig. 1). Even though initially yeast was used to purify interacting proteins, now a variety of organisms are used including plants, as shown by Rohila et al. in (2004) for the first time by using a synthetic TAP-tag which confirmed the interaction of hybrid transcription factor HSP70 and HSP90 similar to the earlier published data.

TAP-MS can be used to study the function of a protein complex responsible in a pathway for development, or stress response by isolating the complex from different biomass sources, for example: protein extraction from specific organs to study stress response. During membrane-bound protein complex purification the subcellular compartment containing the complex is fractioned followed by solubilization of the complex using detergent, maintaining the integrity of the complex. Thus, unlike Y2H, TAP-MS is highly sensitive to membrane- or chromatin-bound proteins (Dedecker et al. 2015). It has two steps for protein complex purification which decrease the background noise meaning reducing the false positive results and identifying transient interacting protein complexes in suspension cultured cells; proteins are highly expressed in abundant amount (Van Leene et al. 2007).

However, TAP-MS also has pitfalls related to high rate of false positive data. Proteins not having binding affinity to the tag, especially the abundant proteins like actin, RuBisCO etc., get co-purified along with the complex increasing the background contamination.

Specific controls or statistical analysis are done to decrease the background contaminants (Zhang et al. 2010, Dedecker et al. 2015). There is also a high probability for identification of proteins that do not interact with each other or study of less abundant protein leading to false negativity. False negativity can be resolved by combining multiple TAP eluates from parallel purifications or increasing the amount of plant extract before purification (Van Leene et al. 2008**).** Purifying proteins using two tags makes the process lengthy. Two-step purification risks the loss of protein example: the weak interactions are not detected because the protein complex is falling apart. Using buffers, salts and stabilizers an artificial surrounding like *in vivo* can be created to maintain the integrity and isolate the complex quickly (Dedecker et al. 2015). Overall, TAP-MS is a simple, high-yield and high-throughput technique for protein-protein interaction study.

The objectives of my study were to make transgenic plants expressing MFP1/cTAPi (gene of interest) construct. Since MFP1 is a chloroplast protein with chloroplast targeting peptide (cTP) at the N-terminus, the TAP tag needed to be added to the C-terminus so that it doesn't block the import into chloroplasts. The next objective was to optimize the Tandem Affinity Purification for the purification of MFP1 complex. Up to date no TAP protocol for purification of thylakoid proteins has been published yet so it will be a new contribution. The final objective is to identify the chloroplast interacting partners of MFP1 that were purified doing mass-spectrometry and finally study the function of MFP1 based on its protein-protein interactions.

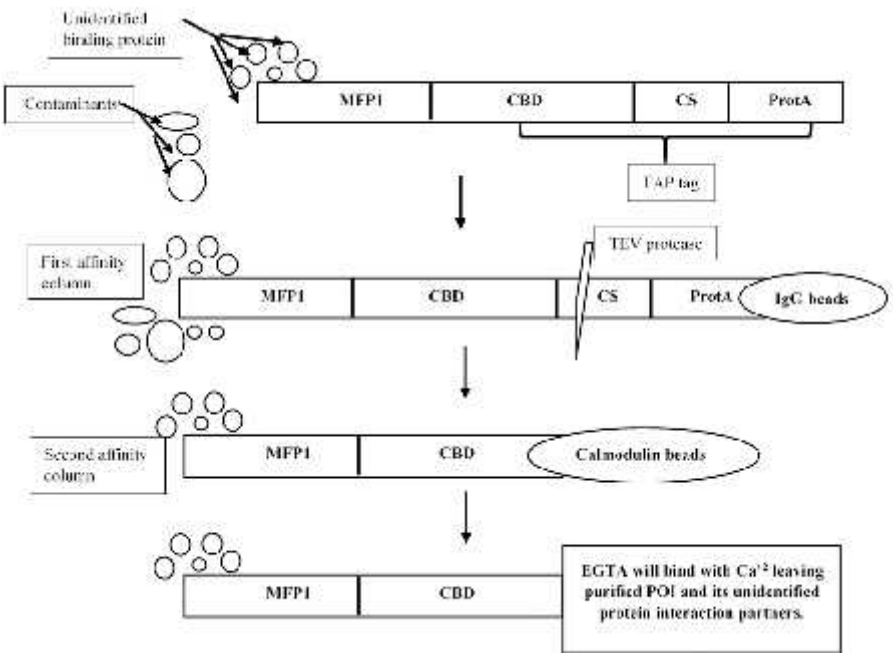

Fig. 1. Tandem affinity purification (TAP): Flow chart of affinity washes and purification steps to obtain unidentified interacting proteins for MFP1. CBD: Calmodulin-binding peptide, CS: TEV protease cleavage site, and ProtA: Protein A.

Materials and Methods

Plants and growth conditions

Two different types of plants were used; WS ecotype wild type (WT) (Fig. 2 A) and K-8-5 mutant (MT) in WS background (Fig. 2 B) *Arabidopsis thaliana*. Seeds were generously provided by the Meier lab at The Ohio State University. The K-8-5 mutant had a T-DNA knockout into the genome lacking MFP1 gene expression (Jeong et al. 2003).

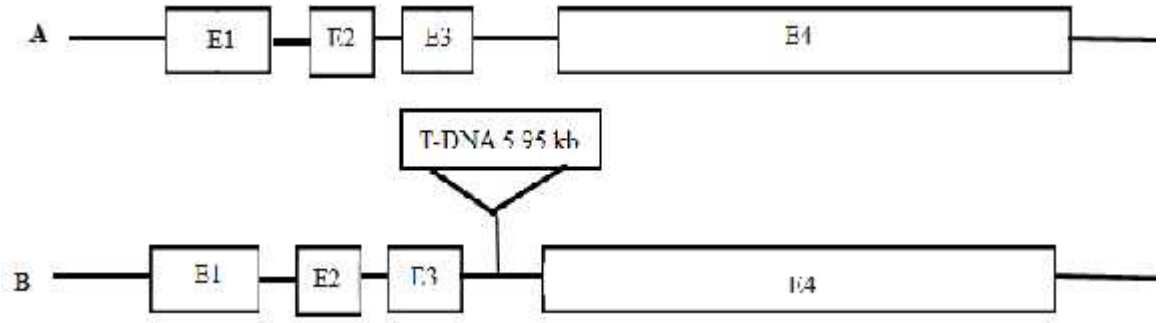

Fig. 2. A: WS ecotype wildtype MFP1 gene, B: K-8-5 mutant MFP1 gene; E1-E4: exons

Seeds were planted on Metro Mix 360 soil (SunGro, Agawam, MA) in 2-inch diameter pots, subjected to cold treatment (2-3 days at 4°C) and transferred to a growth chamber (Percival Environmental Chamber E-30B, Percival Scientific Inc., Boone, IA, USA) under. Seedlings were grown under long-day conditions (16 hours light/8 hours dark, 23°C day temperature, 22°C night temperature) until flowering followed by trimming the flowers from time to time to produce more flowers.

Transformation of *Agrobacterium tumefaciens*

Vector materials

The cTAPi vector was generously provided by the Fromm lab at the University of Nebraska. Two types of cTAPi vector were used as plasmid materials; one cTAPi vector (vector control) which contained only the expression construct for tandem affinity purification (TAP) (Fig. 3 A; Rohila et al. 2004) and the other cTAPi/MFP1 vector (gene of interest) with the expression construct for MFP1 fused with the TAP tag (Fig. 3 B). cTAPi/MFP1 vector was cloned by DeShields (2017). The T-DNA region in the vector is the region that inserts into the *Arabidopsis thaliana* genome. The insertion is confirmed with the help of the BASTA herbicide resistance gene present in the construct. The other remaining region of the vector has a spectinomycin resistance gene. This gene is used to confirm the transformation of *E. coli* and *Agrobacterium tumefaciens*. These vectors were transformed into competent *E. coli* (strain DH5α) cells for vector amplification.

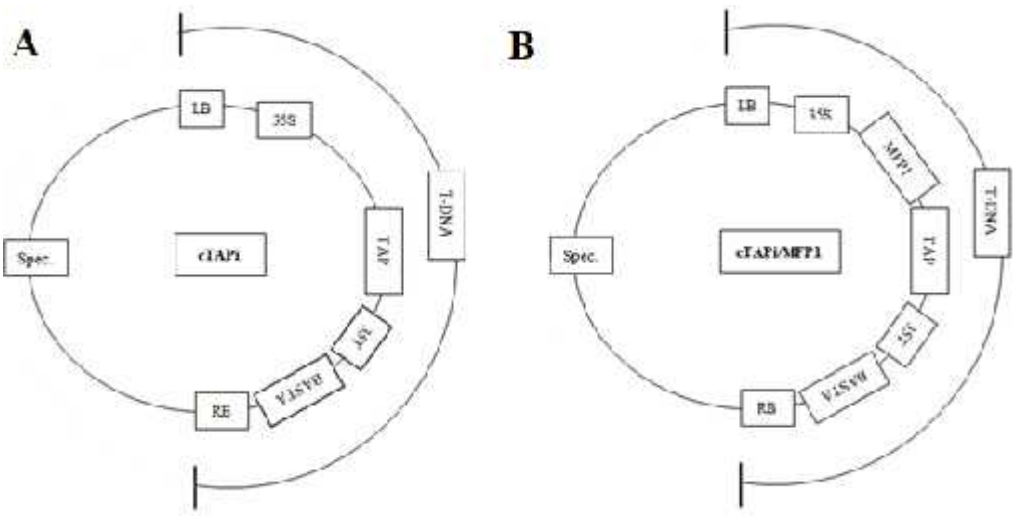

Fig. 3. A: cTAPi and B: cTAPi/MFP1 (MFP1/TAP) vector depicting T-DNA left and right borders (LB, RB), spectinomycin resistance gene (Spec.), Cauliflower mosaic virus 35S promoter (35S), TAP-tag gene (TAP), BASTA herbicide resistance gene (BASTA), and

MFP1 protein of interest (POI). The half circle represents the T-DNA that will be inserted into the plant genome.

Electroporation

Prepared competent *Agrobacterium tumefaciens* GV3101 cells (DeShields, 2017) were transformed with cTAPi/MFP1 and cTAPi plasmid vectors by combining with 1 µl of plasmid DNA (containing about 50 ng of DNA) in a pre-chilled 1 mm gap electroporation cuvette (Eppendorf AG, Hamburg, DEU). The cuvette was then placed into a Multiporator® (Eppendorf AG, Hamburg, DEU) with the mode set to prokaryotes 'O', voltage 1500V, and a time constant of 5 milli secs. Immediately 1 ml of LB medium (5 g Tryptone (IBI Scientific, Peosta, Iowa), 2.5 g Yeast Extract Powder (USB Corporation, Cleveland, OH, USA), 2.5 g Sodium Chloride (USB Corporation, Cleveland, OH, USA), 400 ml deionized water (pH 7.5, adjusted using 1 M Sodium Hydroxide (Sigma-Aldrich, St. Louis, MO) was added to the transformed cells and transferred to a 1.5 ml tube. The tube was incubated (VWR Mini Incubator, VWR International, Bro) at 28ºC for 90 mins. with gentle agitation followed by centrifugation at 3000 x g (5550 rpm) for 1 min. After centrifugation, 900 µl of media was removed and remaining pellets with media in the tubes were resuspended and plated in LB agar plates with antibiotics added for selection (10 µl/ml of Rifampicin (Sigma-Aldrich), 25 µl/ml of Gentamycin (Sigma-Aldrich), 100 µl/ml Spectinomycin (Sigma-Aldrich), and 300µl/ml Streptomycin (Sigma-Aldrich)) and incubated for 2-3 days at 28°C.

Colony Polymerase Chain Reaction

Colony PCR was performed to verify transformation success. Primer sequences (ordered from Eurofins MWG Operon, Huntsville, Alabama) used for PCR and sequencing with melting temperatures (Tm) are presented in Table 1. cTAPi Forward + MFP1 Reverse and MFP1 Forward + cTAPi Reverse primer sets were used for confirming gene of interest

(cTAPi/MFP1) and cTAPi Forward + cTAPi Reverse primer set was used for confirming the control construct (cTAPi).

Table 1. Primer sequences for confirming gene of interest and vector control.

Primer	**Sequence**	**Tm (ºC)**
cTAPi Forward	5'CCTCGGATTCCATTGCCCAGC 3'	64.6
cTAPi Reverse	5'TCGCTTCGGCGAGCAGGTTG 3'	64.6
MFP1 Forward	5'ACTTCAACGATCACTAGGAGAGGCA3'	66.5
MFP1 Reverse	5'ATTGCCTCTTTCATCAGCCAAAGCG3'	66.6

PCR reactions were performed using Taq polymerase (GenScript®, Grand Cayman, KY) according to the manufacturer's recommendations. Each of the PCR reactions contained 1.0 µl (10 µM) of a forward primer and 1.0 µl (10 µM) of reverse primer. Reaction with cTAPi/MFP1 construct contained cTAPi F + MFP1 R and MFP1 F + cTAPi R primers whereas reaction with only TAP construct contained cTAPi F + cTAPi R primers. Each of the reactions also contained 42.0 µl sterile water, 5.0 µl 10x PCR buffer, 0.5 µl 25mM dNTPs (New England Biolabs, Ipswich, MA) and 0.5 µl Taq polymerase. The cycle program for the colony PCR for the amplification of the *Agrobacterium tumefaciens* strain GV3101 had an initial melt at 94°C for 5 mins, followed by 30 cycles consisting of a 1min denaturing step at 94°C, a 1 min annealing step at 57°C, and a 2 mins extension step at 72°C, with a final extension step at 72°C for 5 mins (using a GeneAmp PCR system 9700 thermocycler, Applied Biosystems, Carlsbad, CA). PCR samples were stored overnight at 4°C and checked the next day on a 1% agarose gel (agarose from National Diagnostics, Atlanta, GA, USA) containing 1 µg/ml ethidium bromide (USB) at 100 volts for 1 hour to confirm samples. Gel

images were captured using an AlphaImager HP System (ProteinSimple, San Jose, California, USA).

Transformation of *Arabidopsis thaliana*

Preparation of transformed *Agrobacterium* GV3101 cells for floral dipping

Single colonies of the transformed *Agrobacterium* GV3101 were grown in 3 ml LB medium with the appropriate antibiotics (same as for growing electroporated cells) at 28°C with vigorous shaking until a dense culture was formed (1-2 days). 200 ml of the LB medium with the appropriate amount of antibiotics was inoculated with 2 ml of the small culture and incubated again at 28°C with vigorous shaking until a dense culture was formed (1-2 days). The optical density (cell density) of the transformed *Agrobacterium* GV3101 was measured in a spectrophotometer (Genesys 20, Thermo Scientific, Waltham, MA) at OD_{600}. The cells were harvested by centrifugation at 3,000 x g for 15 mins at room temperature (RT) and resuspended to a final optical density of OD_{600} = 0.8 (0.5-1.0) in resuspension solution (5% Sucrose (Sigma-Aldrich), 0.04% Silwet L-77 (Lehle Seeds)). Four different *Agrobacterium* solutions were made based on the construct and presence or absence of 300 µM Acetosyringone (Sigma-Aldrich) as shown in Table 2 to transform the K-8-5 mutant and WS ecotype wildtype *Arabidopsis thaliana* (Clough and Bent 1998).

Table 2. Different solutions of Agrobacterium used for floral dipping.

Solutions	Gene constructs	Acetosyringone
Solution A	MFP1/TAP	Absent
Solution B	MFP1/TAP	Present
Solution C	TAP only	Absent
Solution D	TAP only	Present

Floral Dipping of *Arabidopsis thaliana*

Agrobacterium-mediated floral dip transformation was used for plant transformation (Clough and Bent 1998). Inflorescences of the MT and WT *Arabidopsis thaliana* were dipped in the respective solutions as listed in Table 2 for 10 secs - 2 mins with gentle agitation and covered in plastic wrap for 24 hours. The trays with the dipped plants were watered and incubated in a growth chamber under long day condition (16 hours light/8 hours dark, 23°C day temperature, 22°C night temperature) until the plants set seeds and were allowed to dry. Finally, seeds were collected from the dried plants.

Plants and Growth Conditions

MT and WT seeds were sterilized with a solution containing 1.2 ml 8.25% Sodium Hypochlorite solution (Clorox brand), 8 µl TWEEN (Sigma-Aldrich), and 8.8 ml deionized water for 10 mins. The seeds were briefly centrifuged in a microcentrifuge at 16,100 x g (Eppendorf Centrifuge 5415 D, Eppendorf AG, 22331 Hamburg, Germany), the sterilization solution was removed, and the seeds rinsed with 500 µl of sterile water 4 or 5 times. Once sterilized, the seeds were placed evenly on Petri plates containing Arabidopsis germination medium (AGM) (1 x Gamborg's B5 (Caisson Labs Inc., North Logan, UT, US), 500 ppm 2-(N-morpholino) Ethanesulfonic Acid (MES) (USB Products, Affymetrix Inc., Santa Clara, CA, USA), Deionized water, (pH 5.7, adjusted using 1 M KOH), 3% w/v Sucrose, Phytoblend (Caisson Labs Inc., North Logan, UT, US) and 10 mg/L BASTA (glufosinate/phosphontricin) (Gold Biotechnology Inc., St. Louis, MO) herbicide. The plates were placed in a growth chamber under long-day conditions (16 hours light/8 hours dark, 23°C day temperature, 22°C night temperature), the seeds allowed to germinate for 7-10 days, and seedlings transferred on soil for further growth.

DNA extraction and polymerase chain reaction analysis

To confirm the plants were transformed, genomic DNA was extracted from leaf tissues using Qiagen DNeasy plant mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. PCR reactions were performed to test transformed plants using the same set of primers and PCR conditions used to confirm the transformed *Agrobacterium* GV3101 cells**.**

The plants that were confirmed transgenic were grown further and finally allowed to dry again for seed collection. The seeds collected this time are heterozygous or homozygous because of segregation and known as the T_1 seeds. T_1 seeds were planted directly in pots using Metro Mix and chilled at 4 ºC for 24-48 hours. The trays were then placed in a growth chamber under long-day conditions (16 hours light/8 hours dark, 23 ºC day temperature, and 22 º C night temperature) and the seeds were germinated and grown.

DNA extraction and PCR analysis of genotype and transgene of T_1 seeds

DNA was isolated manually from the leaf tissues using extraction buffer (100 ml 0.2 M Tris-HCl (USB) at pH 9, 100 ml 0.4 M Lithium Chloride (Sigma-Aldrich), 25 ml 25 mM EDTA (USB), 50 ml 1% SDS (USB), and 225 ml deionized water). The leaves were ground using a pellet pestle in a 1.5 ml centrifuge tube in 500 µl extraction buffer. After centrifuging for 5 mins at 16,100 x g in a microcentrifuge (Eppendorf), the supernatant was removed, spun again, and 400 µl of the cleared supernatant was placed in a tube containing 400 µl isopropanol (Sigma-Aldrich). This was mixed, spun again, the supernatant discarded, and the pellet dried. The pellet was gently suspended in 100 µl of TE buffer by rocking (Maxi-

Rotator), and the DNA was then used for PCR to analyze the genotypes and transgene of the plants.

PCR was performed to verify genotypes of the plants. The primer sequences used for PCR and sequencing with melting temperatures (Tm) are presented in Table 3. The WT allele was amplified with the MFP-FP and MFP-RP primers. Likewise, the MT T-DNA allele was amplified with the JL-202 and MFP-RP primers.

Table 3. Primer sequences for confirming genotype.

Primer	**Sequence**	**Tm (°C)**
MFP1-FP	5'-GGG CTT CTG TGT TCG ATG AAT GTC G-3'	59.5
MFP1-RP	5'-TTC TTA TGA GTT CTT CCT TCT GCT GTT TG-3'	57.1
JL-202	5'-CAT TTT ATA ATA ACG CTG CGG ACA TCT AC-3'	56.2

PCR reactions were performed to test genotype using primers 1) MFP-FP + MFP-RP, and 2) JL-202 + MFP-RP and master mix (Go Taq Green Master Mix, Promega, Madison, U.S.A) containing buffer, Mg^{2+} (cofactor for the enzyme), dNTPs (nucleotides), Taq polymerase enzyme and green loading dye for gel electrophoresis. Each of the PCR reactions contained 1.0 µl (10 µM) forward primer and 1.0 µl (10 µM) reverse primer, 12.5 µl master mix, 2 µl DNA and 8.5 µl sterile water. The cycle program had an initial melt at 94°C for 5 mins, followed by 30 cycles consisting of 30 secs denaturing step at 94°C, 30 secs annealing step at 58°C, and 2 mins extension step at 72°C, with a final extension step at 72°C for 5 mins. PCR samples were stored overnight at 4°C and checked the next day on a 1% agarose gel containing 1 µg/ml ethidium bromide at 100 volts for 1 hour to confirm samples.

Protein expression in T_1 transformed seeds

To confirm transgene expression in the T_1 seeds, total protein was extracted at 4°C from the leaves using protein extraction buffer (20 ml Glycerol (USB), 4 g SDS, 0.757 g Tris, pH 6.8, add sterile water to make final volume 90 ml and filter sterilize) to perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. Right before the use of extraction buffer; 9/10 extraction buffer and 1/10 β-mercaptoethanol (Sigma-Aldrich) were mixed and kept in ice. Liquid nitrogen cooled mortar and pestle were used to grind 0.1 g of leaf tissues into a fine powder. The powder was transferred to a 1.5 ml tubes and resuspended in 0.5 ml extraction buffer (with β-mercaptoethanol). The tubes were incubated in a hot plate (Type 18400 Nuova II Stirring Hot Plate, Barnstead International, Dubuque, Iowa) set at 70°C for 10 mins, transferred to 4°C centrifuge (Eppendorf) and spun at 15,000 rpm for 10 mins. The supernatant with the protein was transferred to a fresh 1.5 ml tube, frozen in liquid nitrogen, and stored at -80°C. SDS-PAGE and western blot analysis was done according to Sambrook and Russell (2001). Two identical gels were run using 10% and 5 % polyacrylamide for the running and stacking gel mixture using Bio-Rad Mini Protean Tetra System (Bio Rad, Hercules, CA) as the gel tank for Coomassie staining and western blotting. For the western, transfer was done using Mini Trans Blot (Bio-Rad Laboratories, Hercules, CA); following the blot 1x Ponceau staining confirmed protein transfer to the nitrocellulose membrane (Bio-Rad Trans-Blot Transfer Medium). Two different antibodies, MFP1 (OSU91; 1:5,000) (generously donated by the Meier lab at the Ohio State University) and TAP (1:1,000) (Thermo Fisher, Life Technologies Corp., Carlsbad, CA), were tested

using the same secondary antibody anti-rabbit IgG, horseradish peroxidase (1:10,000) (GE Healthcare UK Limited, Little Chalfont Buckinghamshire). Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) was used on blots for detection under ChemiDoc MP Imaging System (Bio Rad Laboratories, Hercules, CA).

Chloroplast isolation

Chloroplast isolation was performed using a protocol modified from Kügler et al. (1997). 15 g leaf tissue collected from 8-week-old plants was ground in 150 ml ice-cold extraction buffer (330 mM Mannitol (USB), 30 mM HEPES (Calbiochem, EMD 14 Millipore, Darmstadt, Germany), 3 mM Magnesium Chloride (Sigma-Aldrich), 2 mM EDTA, 0.1% (w/v) BSA (Sigma-Aldrich)) using a polytron homogenization (Polytron system PT 10-35 GT, Kinematica AG, Switzerland) for 3 x 5 secs. The homogenized tissue was filtered through four layers of Miracloth (Calbiochem) to remove leaf debris. The filtrate was centrifuged at 4°C at 2,000 x g for 3 mins in a swinging bucket rotor (Sorvall Legend XTR, rotor 3607, Thermo Scientific). After centrifugation, the supernatant was discarded, and the pellet was resuspended in 3 ml extraction buffer. The resuspended pellet was carefully pipetted onto 25 ml 40% v/v Percoll (Research Organics, Cleveland, OH) solution in extraction buffer, then centrifuged for 30 mins at 4,700 x g in a swinging bucket rotor (Legend XTR, rotor 3607) with the brakes deactivated to prevent the mixing of the two layers. The intact chloroplasts, located in the bottom layer as shown in Fig. 4, were recovered and washed with 20 ml resuspension buffer (extraction buffer without BSA), gently mixed by inversion, and recentrifuged at 2,500 x g for 10 mins. This step was repeated twice, and the

supernatant was discarded. The intact chloroplast suspension was split into 500 µl aliquots and stored in -80ºC.

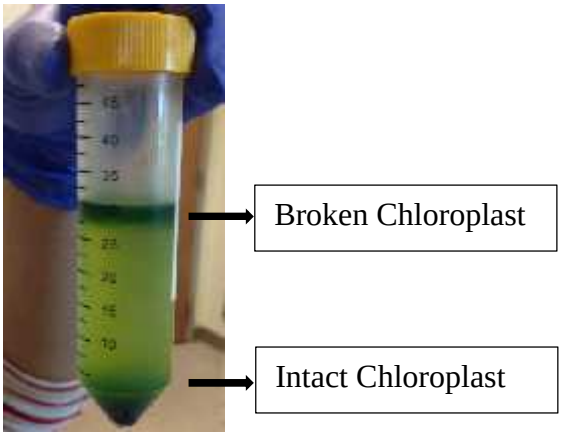

Fig. 4. Isolation of intact chloroplast: The bottom dark green layer consists of the intact chloroplast and the upper layer consists of the broken chloroplast isolated from fresh leaf tissues of transgenic Arabidopsis plants.

Tandem Affinity Purification (TAP) purification for MFP1 complex purification

Proteins were extracted from the chloroplast pellet first and purified by TAP using three different detergents; digitonin, Triton X-100 and n-dodecyl -D-maltoside (DDM) in the buffer following Andrès et al. 2011.

Use of Digitonin as detergent

Protein was extracted from chloroplast pellets by resuspending the pellet in 160 µl solubilization buffer (50 mM Bis-Tris-HCl (Amresco Inc., Solon, OH, USA), pH 7, 0.5 M Aminocaproid Acid (Spectrum Chemical, New Brunswick, NJ, USA), 10% w/v Glycerol, 1% w/v water-soluble Digitonin (EMD Millipore), 1% (v/v) Plant Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO)). The suspension was incubated for 10 mins, centrifuged at 16,100 x g for 10 mins, and supernatant was transferred to a fresh 1.5 ml tube. Supernatant was incubated with 150 µl HsIgG beads (IgG Sepharose 6 Fast Flow, GE Healthcare,

Uppsala, Sweden) (10 µl of HsIgG beads per g of initial fresh tissue) for overnight at 4°C with gentle rotation. After centrifugation at 100 x g for 1 min at 4°C, the HsIgG beads were recovered and washed twice with the same volume of ice-cold solubilization buffer used in chloroplast isolation. The beads with the solubilization buffer were transferred into the spin column (Mobitec). The beads were washed 5x with ice-cold solubilization buffer using 50 volumes of ice-cold solubilization buffer. For each 10 µl of beads, 1.5 µl of 20 x TEV buffer and 5U of rTEV protease (Invitrogen, Life Technologies Corp., Carlsbad, CA), 0.1 M Dithiothreitol (DTT, USB), 5% w/v water-soluble Digitonin and 30 µl of ultra-pure water were added and incubated overnight at 4°C. The spin column was placed into a new 1.5 ml tube and spun at 100 x g for 1 min at 4°C. In the same column 100 µl of cold 1 x TEV buffer (Invitrogen), 0.1 M DTT, 5% w/v water-soluble Digitonin (Invitrogen) were added and spun at 100 x g for 1 min at 4°C to collect the TEV eluate. To the TEV eluate: 1 volume of Calmodulin-binding buffer (CBB, 10 mM Tris-HCl, pH 7.9 (VWR International, Sigma-Aldrich), 100 mM Sodium Chloride, 2 mM Calcium Chloride (USB), 10 mM β-mercaptoethanol, 5% (w/v) water soluble Digitonin (Invitrogen)) and 3 µl of 1 M Calcium chloride were added and transferred to a spin column containing Calmodulin Agarose Beads (Calmodulin Sepharose 4B, GE Healthcare) (1 µl of Calmodulin Agarose beads per 2 µl of HsIgG beads) and incubated overnight on turning plate at 4°C. The beads were washed with 500 µl of CBB followed by 300 µl of Calmodulin Washing Buffer (CWB, 10 mM Tris-HCl, pH 7.9, 100 mM Sodium Chloride, 0.1 mM Calcium Chloride, 10 mM β-mercaptoethanol, 5% (w/v) water soluble Digitonin) by spinning the column in a new collection tube. Finally, the protein was eluated using Calmodulin Elution Buffer (CEB, 10 mM Tris-HCl, pH 7.9, 100 mM Sodium Chloride, 10 mM β-mercaptoethanol, 5% (w/v) water soluble Digitonin,

100-200 mM Potassium Acetate (VWR International), 5-20 mM Ethylene glycol-bis (2-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA, Sigma-Aldrich)). Each time the spin column was placed in a new 1.5 ml tube by adding 100 µl of CEB and incubating for 3 mins each followed by spinning at 100 x g for 1 min at 4 ºC.

Use of Triton X-100 as detergent

Protein extraction from chloroplast pellets and TAP of the MFP1 complex was done by following the protocol as described above but replacing solubilization buffer with grinding buffer (100 mM Sodium Chloride, 50 mM Tris-HCl, pH 7.9, 0.5% (v/v) Triton X-100 (USB), 1 mM phenylmethanesulfonyl fluoride (PMSF, USB), 5 mM Sodium Floride (J.T. Baker, Avantor Performance Materials, Inc. Center Valley, PA), 0.2% (v/v) Plant Protease Inhibitor Cocktail)) and using 0.5% (v/v) Triton X-100 in place of 5% (w/v) water soluble Digitonin. Triton X-100 was also used as a detergent to prepare buffers in the TAP protocol.

Use of n-dodecyl-D-maltoside (DDM) as detergent

Protein extraction from chloroplast pellets and TAP of the MFP1 complex was done by following the protocol as described above for Triton X-100 using 0.5% (v/v) DDM (Sigma-Aldrich) in place of 0.5% (v/v) Triton X-100. DDM was also used as a detergent to prepare buffers in the TAP protocol.

TAP purification procedure based on Rubio et al. 2005 with some modifications

Protein was extracted following the protocol described above from chloroplast pellets by resuspending the pellet in 200 µl of extraction buffer (50 mM Tris–HCl pH 7.5, 150 mM Sodium Chloride, 10% Glycerol, 0.1% Nonidet P-40 Alternative (Sigma-Aldrich), 1

mM PMSF, and 1x complete protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The protocol listed in Rubio et al. (2005) was followed for extract incubation with the beads in the first and second purification steps, and TEV cleavage was done overnight with 5 units of rTEV protease in cleavage buffer. After the cleavage, supernatant containing the MFP1 complex was recovered and incubated with 125 µl of Calmodulin beads at 4°C with gentle rotation overnight. After centrifugation at 150 x g for 3 mins at 4°C, the beads were washed with 5 ml washing buffer and elution was performed using imidazole containing buffer (50 mM Tris–HCl pH 7.5, 150 mM Sodium Chloride, 10% Glycerol, 0.1% Nonidet P-40 Alternative, 0.05 M Imidazole (USB)).

TAP purification procedure based on Rohila et al. 2006 with some modifications

Protein was extracted from chloroplast pellets by resuspending the pellet in 200 µl of extraction buffer (20 mM Tris–HCl, pH 8.0, 150 mM Sodium Chloride, 0.1% NP-40 Alternative, 2.5 mM EDTA, 2 mM Benzamidine (Sigma), 10 mM β-mercaptoethanol, 20 mM Sodium Fluoride, 1 mM PMSF, 1% Plant Protease Inhibitor Cocktail (Sigma-Aldrich), 10 µM Leupeptin (Sigma-Aldrich)), and incubated for 10 mins. The suspension was centrifuged at 16,100 x g for 10 mins and supernatant was transferred to a fresh 1.5 ml tube. For the TAP, the protocol from Rohila et al. (2006) was followed but 0.1% NP-40 Alternative was used as detergent and simple 1.5 ml tubes were used instead of polyprep chromatography column for washing steps.

Final purification of MFP1 complex and protein isolation was confirmed doing SDS-PAGE, and western blot and Coomassie stain respectively as described in protein expression of T_1 transformed seeds.

Transmission Electron Microscopy

To study the thylakoid membrane structure in wildtype, mutant, and transgenic plants, Transmission Electron Microscopy (TEM) was done. Leaf samples taken from four-week-old plants were cut into fragments (≤ 3 mm wide) and fixed in a 2.5% solution of glutaraldehyde (SPI Supplies, West Chester, PA) in a 1 mM phosphate-buffered saline (pH 6.7) (PBS, Sigma-Aldrich), for 3 days to ensure proper tissue infiltration. After 3 days, the glutaraldehyde solution was removed, and the samples were washed thrice for 10 mins with PBS. Samples were post-fixed using 1% osmium tetroxide (Ted Pella, Redding, CA) in PBS for 1 hour, then washed thrice for 10 mins in PBS, followed by dehydration in an ethanol (Sigma-Aldrich) gradient (successively, 50% ethanol for 2 hours, followed by 70%, 85%, 95% and 100% ethanol for 1 hour and 15 mins each, then again in 100% ethanol overnight). Samples were removed from ethanol and soaked twice for 10 mins in 100% propylene oxide (Electron Microscopy Sciences, Hatfield, PA). A 1:1 mixture of propylene oxide and Spurr's Low-Viscosity Resin (SPI Supplies) was prepared, and the samples were infiltrated in the mixture overnight with the bottles uncapped to evaporate the propylene oxide. The next day, the old resin was removed with enough new epoxy resin added to cover the samples. The leaf tissue was embedded in flat embedding molds (BEEM brand, Ted Pella) and cured at 65°C for 14-15 hours. Once hardened, the samples were trimmed in a trapezoid shape with a razor just enough to expose approximately 3 mm of sample. The end of the sample was also

trimmed to ensure that the tissue sample was exposed and would be present in microtome slices. Once trimmed, samples were cut on a microtome (Ultracut E Ultramicrotome, Reichert-Jung, Depew, NY) using a glass knife.

Initially, sections were cut at 1 µm, to ensure that the sample face was flat, that the tissue was exposed, and to generate sections to examine under a light microscope. Once the sample face was flat, 99 nm sections were cut for electron microscopy. These 99 nm sections were then placed on 3 mm copper grids and placed on a piece of filter paper to dry. 1% uranyl acetate (UA, Electron Microscopy Sciences) and 1% lead citrate (Electron Microscopy Sciences) were used to stain the thin sections. The grids were washed 10 x 5 mins in distilled water after the UA and lead staining, respectively, and left overnight to dry. After drying samples, they were examined under a JEM1400 transmission electron microscope (JEOL Ltd., Peabody, MA).

Results

Transformation of *Agrobacterium tumefaciens*

Agrobacterium was transformed with three different plasmids and plated in LB medium with appropriate antibiotics for selection of the transformed cells. *Agrobacterium* transformed with cTAPi/MFP1 (gene of interest) construct showed few colonies, *Agrobacterium* transformed with cTAPi (control) construct showed a larger number of colonies, and *Agrobacterium* cells without DNA added used as control showed no growth as expected when cultured in LB medium with appropriate antibiotics for selection of the transformed cells as shown in Fig 5.

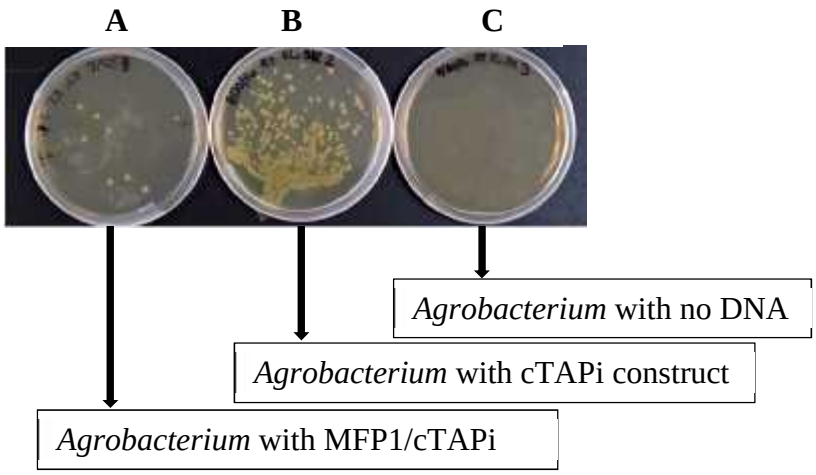

Fig. 5. Selection of transformed *Agrobacterium* cells on LB medium with antibiotics. A) cTAPi/MFP1 (gene of interest), B) cTAPi (control) construct and C) negative control (no DNA).

Transformed *Agrobacterium* cells were further confirmed by colony PCR. *Agrobacterium* transformed with cTAPi/MFP1 construct was confirmed using two sets of primers: 1) cTAPi F + MFP1 R and 2) MFP1 F + cTAPi R, which amplify fragments of 707 bp and 870 bp respectively. The bands in lanes 1 and 2 of Fig 6. showed the band size as estimated. Thus, the appearance of these two bands in the gel confirmed the transformation of *Agrobacterium* with the gene of interest. *Agrobacterium* transformed with cTAPi control construct was confirmed using one set of primers: 3) cTAPi F + cTAPi R, which amplify a fragment of 3079 bp. The band in lane 3 of Fig 6. showed the correct size confirming the transformation.

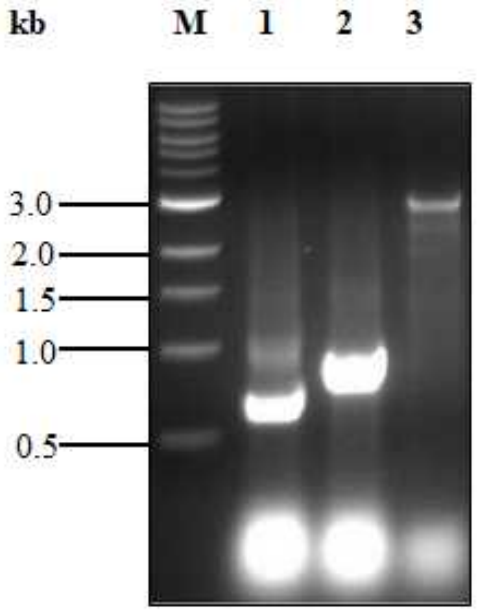

Fig. 6. Colony PCR of transformed *Agrobacterium*: M. 1 kb marker; 1. cTAPi F + MFP1 R primer set confirming MFP1-TAP construct; 2. MFP1 F + cTAPi R primer set confirming MFP-TAP construct; 3, cTAPi F + cTAPi R primer set confirming vector control. Bands confirm that the *Agrobacterium* cells are transformed with the MFP1/cTAPi fusion gene T-DNA construct or vector control, respectively.

Transformation of WS ecotype wildtype (WT) and K-8-5 mutant (MT) *Arabidopsis thaliana*

Wildtype and mutant Arabidopsis plants were transformed with cTAPi/MFP1 or cTAPi control simply by dipping flowering plants in a suspension of transformed bacteria with or without the elicitor acetosyringone. After the floral dip (T_0) seeds were collected from the plants and grown on Petri plates with plant selection medium using BASTA for the selection of transformed plants. Transformed seedlings are BASTA resistant and stay green, while untransformed seedling should be sensitive and turn yellow as shown in Fig 7.

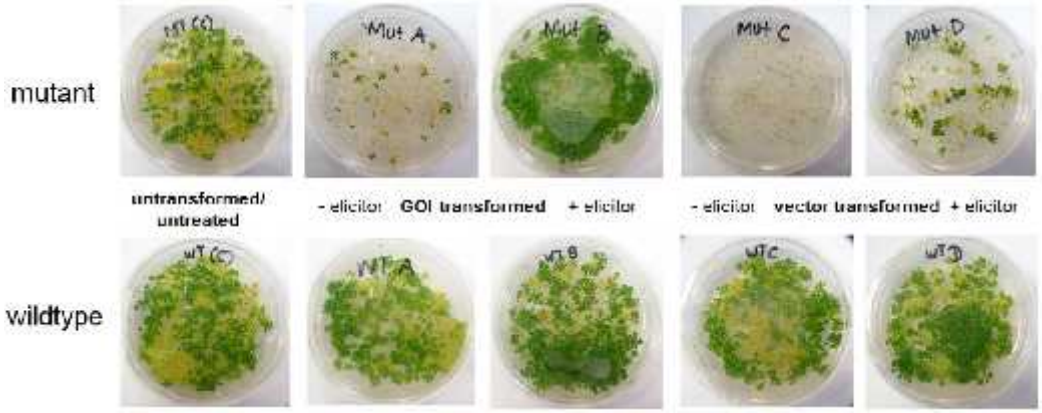

Fig. 7. One-week old T_0 seedlings grown under long day conditions in BASTA plates. Top row, K-8-5 mutant plants; bottom row, WS wildtype plants. Left plates: Untransformed plants used as selection control; GOI transformed (A and B), transformed with MFP1/cTAPi construct; vector transformed (C and D), transformed with vector control. Transformations A and C were performed without elicitor (Acetosyringone), B and D with elicitor. Mutant plants appear to perform poorly after transformation unless complemented with MFP1/cTAPi.

The untransformed controls showed a high background of BASTA resistance. Further selection of the T_0 seedlings was done by PCR using the same set of primers that were used to confirm the transformed *Agrobacterium*. The bands shown in the lanes of Fig. 8 did show the band size as estimated, thus confirming the transformation of *Arabidopsis* with the gene of interest.

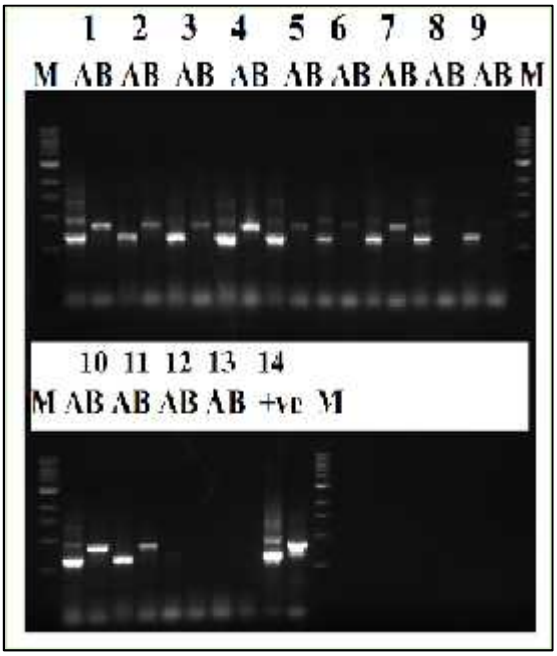

Fig. 8. PCR testing of T1 generation of transformed K-8-5 *Arabidopsis thaliana*: Numbers indicate individual plants, with two PCR reactions performed per plant. A, PCR with cTAPi F + MFP1 R primers; B, PCR with MFP1 F + cTAPi-R primers; +ve, positive control and M, 1 kb marker.

During transformation the elicitor acetosyringone was used to test whether it can increase the efficiency of the transformation process. Plants dipped in solution with transformed bacteria containing acetosyringone showed 41.4% transformation efficiency compared to only 1.6% without acetosyringone confirming that acetosyringone increases the efficiency of the transformation of *Arabidopsis* plants (Table 4).

Table 4. Effect of acetosyringone on transformation efficiency.

ID	Genotype	Construct	Acetosyringone (AS)	Plants analyzed	Transgenic plants	% Transgenic plants
Control	WT	-	-	8	0	0.0
A	WT	MFP1/cTAPi	-	26	0	0.0
B	WT	MFP1/cTAPi	+	22	7	31.8
C	WT	cTAPi	-	16	1	6.3
D	WT	cTAPi	+	18	8	44.4
Control	MT	-	-	8	0	0.0
A	MT	MFP1/cTAPi	-	19	0	0.0
B	MT	MFP1/cTAPi	+	37	28	75.7
C	MT	cTAPi	-	0*	0	N/A
D	MT	cTAPi	-	34	3	8.8
			Total	188	47	25.0
			-AS	61	1	1.6
			+AS	111	46	41.4

* No BASTA-resistant seedlings were recovered.

Genotyping of WS ecotype wildtype (WT) and K-8-5 mutant (MT) *Arabidopsis thaliana*

Due to the possibility of cross-pollination when growing wildtype and mutant plants in the same growth chamber, the transformed plants may be heterozygous or homozygous in genotype. Therefore, PCR using two sets of primers was done to confirm the genotype. The 1) MFP-FP + MFP-RP set of primers was used to amplify the wildtype allele whereas 2) JL-202 + MFP-RP set of primers was used to amplify the mutant allele, as the JL-202 sequence is derived from the T-DNA insertion. All the samples produced bands for JL-202 primer, but not for the MFP1-FP primer (Fig. 9) confirming that the transgenic plants are homozygous for the T-DNA insertion allele.

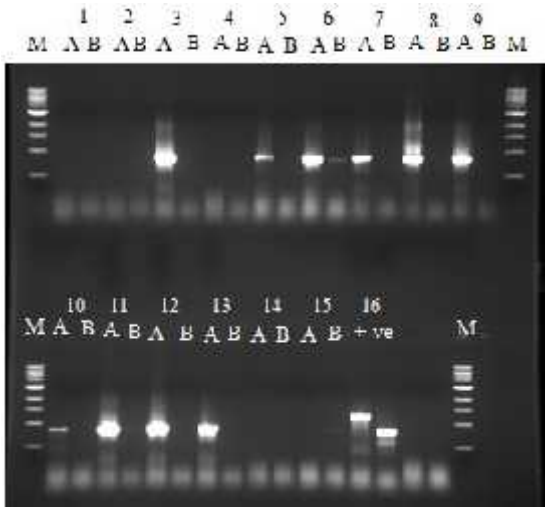

Fig. 9. PCR testing for genotype of T1 generation: Numbers indicate individual plants, with two PCR reactions performed per plant. A, PCR with MFP-FP + MFP1-LP primers; B, PCR with Jl-202 and MFP1-LP primers; +ve, positive control and M, 1 kb marker.

Expression of TAP-tagged MFP1 protein

The expression of MFP1-TAP fusion protein was confirmed by SDS-PAGE and western blotting followed by immuno-detection using the antibody against MFP1 and the TAP tag, respectively. Fig. 10A shows the result of using the antibody against MFP1. Protein extracted from the mutant in the MT lane of Fig. 10A contains no detectable MFP1, wildtype protein in the WT lane of Fig. 10A shows MFP1 of normal 82 kDa size, and the bands in 1-6

are protein extracts from the transgenic lines showing bands of MFP1 fused with the TAP tag with increased molecular weight compared to the normal MFP1 band in WT. These results confirm the expression of tagged MFP1 protein in the transgenic plants.

Fig. 10B shows the result of using the antibody against the TAP tag. The same blot was used to test with the TAP antibody after using stripping buffer to remove MFP1 antibody overnight. No bands can be seen in the MT and WT lane of Fig. 10B as they contain no TAP tag. The bands in lanes 1-6 are the transgenic lines showing bands of TAP fused to MFP1. These results further confirm the expression of TAP-tagged MFP1 protein in the transgenic plants.

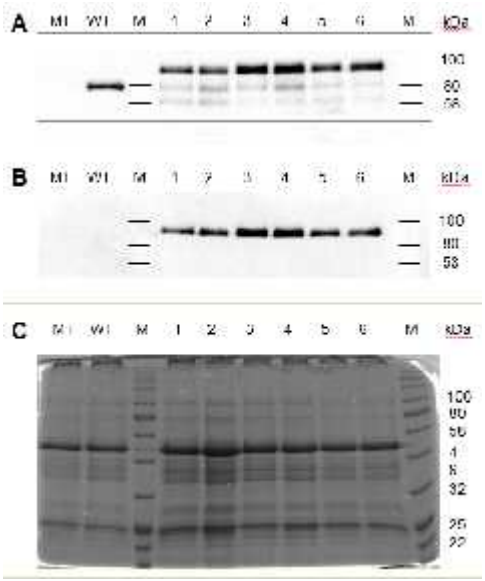

Fig. 10. SDS-PAGE and immunoblotting of selected lines: A, immunoblot using anti-MFP1 antibody. MT, mutant; WT, wildtype; M, NEB pre-stained protein marker; 1-6, transgenic lines. B, immunoblot using anti-TAP antibody. The same samples as for A; signal in 1-6 confirms TAP tag fused to MFP1. C, Coomassie-stained gel. Total leaf protein for samples loaded in A and B.

Localization of MFP1 inside chloroplast

To confirm MFP1 localization inside the chloroplast, intact chloroplasts were isolated followed by protein isolation using digitonin in the extraction buffer. The localization of MFP1 inside the chloroplast was confirmed by SDS-PAGE and western blotting followed by immuno-detection using the antibody against MFP1 as shown in Fig. 11. Bands from lane 3 and 5 as shown in Fig. 11 below show MFP1 in the pellet collected after centrifuging whole leaf extract and removing the supernatant, and in the intact chloroplast, thus confirming the localization of MFP1 inside the chloroplast. The multiple bands may be due to the degradation of the MFP1 during its isolation.

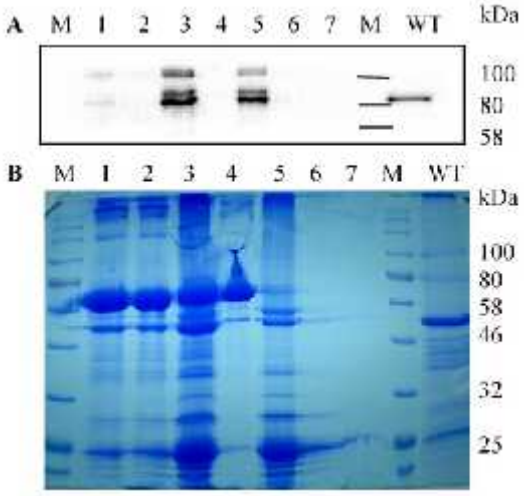

Fig. 11. SDS-PAGE & Western for localization of MFP1: A: immunoblot using anti-MFP1 antibody. M: NEB pre-stained colored protein marker, 1: whole leaf extract, 2: supernatant of leaf extract, 3: pellet of the leaf extract, 4: broken chloroplast, 5: intact chloroplast, 6: IgG flow through, 7: IgG beads and WT: positive control. B: Coomassie-stained gel.

Study of thylakoid membrane

MFP1 is anchored into the thylakoid membrane inside the chloroplast. Transmission Electron Microscope (TEM) was used to view if expression of the tagged protein inside the mutant Arabidopsis has changed the stacking of the thylakoid membrane. There was no

difference observed in the thylakoid structure between the WT, MT and the transgenic plants (Fig. 12).

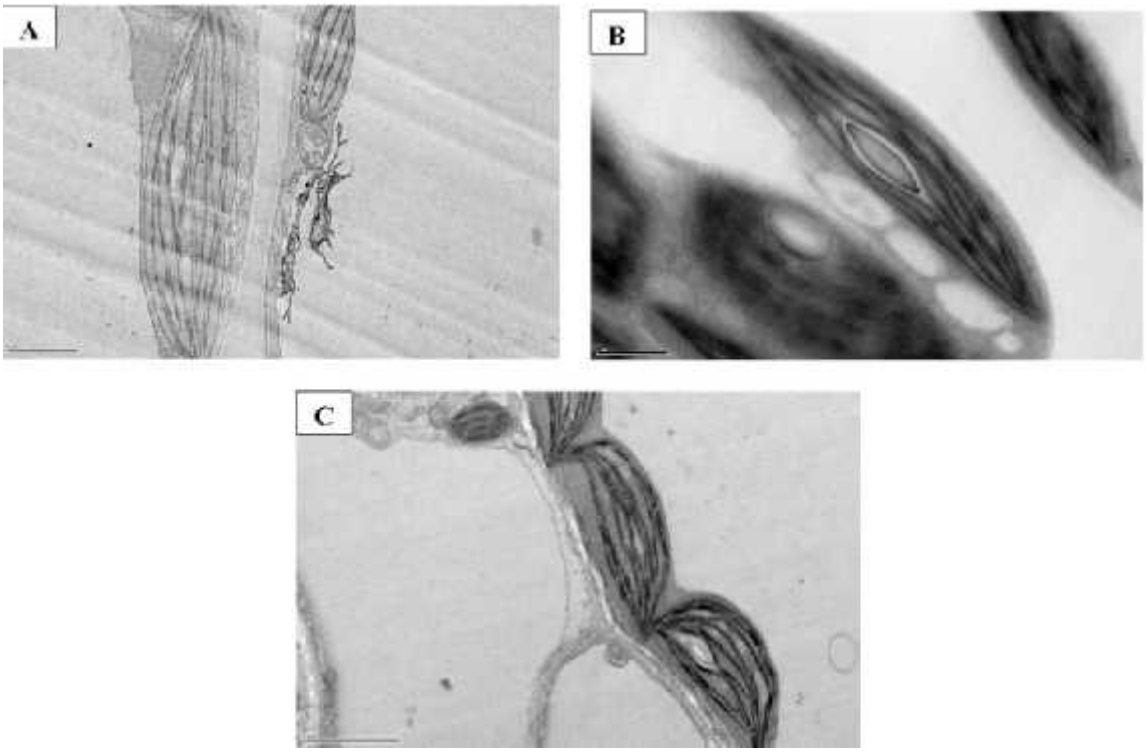

Fig. 12. TEM micrograph of WT (A), MT (B) and transgenic plants (C) chloroplast. Bar. 1µm

Use of digitonin, Triton X-100 and n-dodecyl-D-maltoside (DDM) as detergent for Tandem Affinity Purification

Since MFP1 is a thylakoid membrane protein, solubilization with mild detergent is necessary for the TAP procedure. Three different detergents, digitonin, Triton X-100 and DDM, were used in the extraction, TEV, and Calmodulin buffers for the tandem affinity purification of MFP1 complex following Andrès et al. 2011. The purification of MFP1 complex was checked by SDS-PAGE and western blotting followed by immuno-detection using the antibody against MFP1. No band was seen in the final eluate, thus the purification with these detergents was not successful (data not shown).

Use of NP-40 Alternative as detergent for Tandem Affinity Purification

To successfully purify the MFP1 complex from the chloroplast, the Rubio et al. (2005) protocol was used with some modifications. Initially, MFP1 was bound with IgG beads and confirmed by SDS-PAGE and western blotting followed by immuno-detection using the antibody against MFP1 (Fig. 13). Bands in the lanes 1, 2 and 3 confirmed the presence of MFP1 inside the chloroplast whereas bands in the lanes 4, 5 and 6 confirmed the binding of tagged MFP1 to IgG beads, but multiple bands were present as shown in Fig. 13.

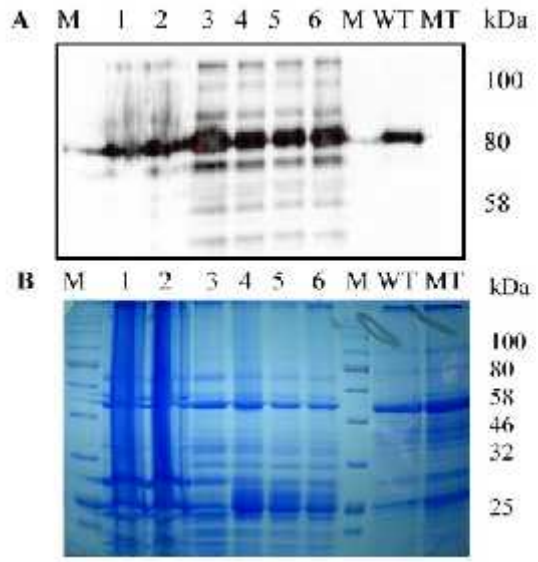

Fig. 13. SDS-PAGE & Western for MFP1 binding with IgG beads using NP-40 Alternative: A: immunoblot using anti-MFP1 antibody. M: NEB pre-stained colored protein marker, 1: Intact chloroplast, 2: Intact chloroplast, 3: Supernatant from lysed chloroplast, 4: protein sample incubated with IgG beads, 5: protein sample incubated with IgG beads, 6: IgG beads, WT: positive control and MT: negative control. B: coomassie-stained gel.

Based on the success with the NP-40 Alternative, the complete TAP method was done following Rubio et al. (2005) for MFP1 complex purification and confirmed doing western using MFP1 antibody (Fig. 14). The protein bands in lane 1-4 confirm the purification of MFP1 using IgG beads and bands in lane 5 confirm the MFP1 after TEV cleavage. Since the protein was degraded in the purification process smaller bands were seen during the TEV cleavage step. However, the protein gets lost after Calmodulin beads incubation.

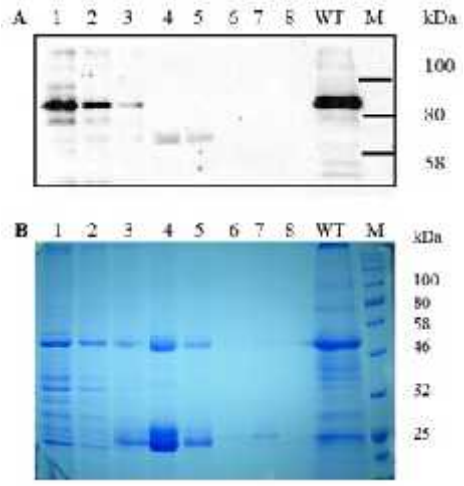

Fig. 14. SDS-PAGE & Western for MFP1 using NP-40 Alternative: A: immunoblot using anti-MFP1 antibody. M: NEB pre-stained colored protein marker, 1: Supernatant collected after chloroplast lysis, 2: Supernatant after incubating with IgG bead, 3: IgG beads, 4: IgG beads after TEV incubation, 5: TEV cleavage, 6: Supernatant after washing the IgG beads with TEV incubation, 7: Supernatant after Calmodulin beads incubation, 8: Final eluate and WT: positive control. B: coomassie-stained gel.

In an attempt to prevent protein degradation, MFP1 complex was purified following Rohila et al. (2006) with the use of E-64 protease inhibitor. E-64 is an irreversible, highly specific, cell permeable and low toxicity protease inhibitor making it useful for *in vivo* studies. It can be used for affinity purification without inhibiting TEV protease, therefore it was safe to use for the TEV cleavage step. MFP1 bands in all the lanes including final eluate as shown in Fig. 15 confirm the purification of MFP1 complex. However final protein concentration was too low to submit the sample for mass spectrometry.

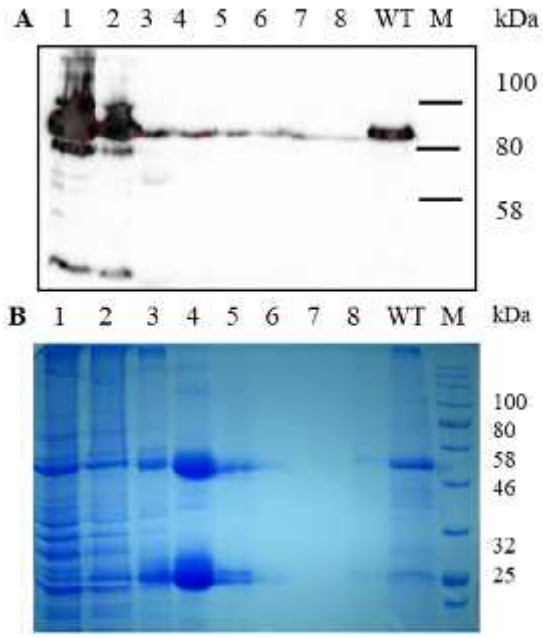

Fig. 15. SDS-PAGE & Western for MFP1 using NP-40 Alternative: A: immunoblot using anti-MFP1 antibody. M: NEB pre-stained colored protein marker, 1: Supernatant collected after chloroplast breakdown, 2: Supernatant after incubating with IgG bead, 3: IgG beads, 4: TEV eluate, 5: Calmodulin beads, 6: Final eluate 1, 7: Final eluate 2, 8: Final eluate 3 and WT: positive control. B: coomassie-stained gel.

Discussion

Different biological functions in a cell are carried out by protein-protein interactions (PPIs) forming a complex. The interactions are conserved in evolution and often mediated by coiled-coil domains which can be exploited to identify novel interactions between proteins (Mier et al. 2017). Since MFP1 largely contains α-helical coiled-coil domains, it should be involved in protein complex formation to perform a function. MAF1 interacts with MFP1 associated with the nuclear matrix located at the nuclear envelope (Gindullis et al. 1999). MFP1 has a dual localization in nuclear matrix and chloroplast but is also found at high or even higher relative concentrations in chloroplast (Majeran et al. 2012). But, MFP1 interacting proteins have not yet been isolated and identified from chloroplasts. The goal of my project is to identify chloroplast interaction partners of MFP1 using TAP-MS to determine the function of MFP1.

To identify chloroplast-localized binding protein partners of Arabidopsis MFP1, I successfully transformed *Agrobacterium* GV3101 cells with cTAPi/MFP1 (gene of interest) which can be used in combination with mass spectrometry (MS) to identify chloroplast protein-protein interaction complexes. The TAP tagging method is an efficient system for purifying protein interaction partners *in vivo* (Xu et al. 2010). I also transformed *Agrobacterium* GV3101 cells with cTAPi as a control which might be useful to identify unspecific proteins binding to the TAP tag. The transformed *Agrobacterium* GV3101 cells were selected by culturing in LB medium with appropriate concentration of different antibiotics (Fig. 5). The vector used to transform *Agrobacterium* GV3101 cells has a spectinomycin resistance gene used to confirm the transformation of *Agrobacterium*

tumefaciens GV3101. Colony PCR was done to confirm the presence of intact T-DNA using two sets of primers, 1) cTAPi/MFP1 (gene of interest) and 2) cTAPi (control) resulting in PCR products of 707 bp and 870 bp which are shown in the lane marked as 1 and 2 of Fig. 6. In the gel picture, additional faint bands can be seen in the lane using the cTAPi F + MFP1 R set of primers due to a dual 35S promoter present in the T-DNA which provides two binding sites for cTAPi F.

Four different solutions of transformed bacterial cells in presence and absence of the elicitor acetosyringone as listed in Table 2 were used as the basis for plant transformation to generate transgenic lines expressing TAP-tagged AtMFP1 to isolate AtMFP1-containing protein complexes from chloroplasts. Acetosyringone is a chemical which acts as an elicitor by mimicking a wounding response which leads to increased infection rate and induces expression of the *vir* genes encoded by the Ti (tumor-inducing) plasmid which facilitate T-DNA excision and transfer by *Agrobacterium* (Veluthambi et al. 1989; Godwin et al. 1991). Thus, I wanted to test whether addition of acetosyringone increases the transformation efficiency of our plants or not. For expressing a tagged protein of interest at a high rate, plants were transformed via *Agrobacterium tumefaciens* mediates floral dipping following the protocol published by Clough and Bent in 1998. *Agrobacterium tumefaciens* introduces the T-DNA with the gene coding for the tagged protein of interest through random integration in the nuclear genome. Selection of the transformed seedlings was done on BASTA (10 mg/l) plates shown in Fig. 7. Transformed seedlings are BASTA resistant and stay green in color, while untransformed seedlings are BASTA sensitive and turn yellow. In untransformed mutant and wildtype plates I found growth of both green and yellow seedlings which made the BASTA selection unsuccessful. This might be because the untransformed

plants have natural resistance. For later study, I would prefer to use herbicide of higher concentration or spraying of herbicide from time to time. The wildtype plates showed similar numbers of green and yellow for all treatments. However, on mutant plates the numbers differed comparing the transformed mutant and wildtype plates. I saw fewer green seedlings in the mutant plates than wildtype except for the mutant transformed with gene of interest. This may indicate that the mutant after dipping in the pathogen showed slow maturation resulting in fewer seeds and thus fewer seedlings, and transformation with the cTAPi/MFP1 (cDNA) can complement this phenotype.

Because BASTA selection was not reliable, identification of transformed plants was done using PCR on green seedlings with the same set of primers as for *Agrobacterium* colony PCR. As expected bands of 707 and 870 bp were obtained representing the insertion of gene of interest inside the plants as shown in Fig. 10. Looking at the PCR results I found the transformed bacterial solution treated with acetosyringone showed high efficiency of transformation 41.4% (plates B and D of mutant) (Table 4). Thus, my result matched up with the finding done by Godwin et al. in 1991.

Following the insertion of the gene of interest, the expression of the MFP1-TAP fused protein in the transgenic plants was confirmed by SDS-PAGE and western blot. Untagged MFP1 is 82 kDa in size which was shown in the wildtype plant when the antibody against MFP1 was used. The transgenic samples showed a band size of around 94-100 kDa a little bit higher than 82 kDa in size as expected because MFP1 in the transgenic samples is fused with the TAP tag (Fig. 10A). In addition to the bands of interest, I also got some faint bands. This might be because of degradation of the protein samples. When the same blot was used for

testing the expression of TAP in these samples I got the same band patterns confirming the expression of TAP along with full-length MFP1 in the transgenic plants (Fig. 10B). No signal was observed for the smaller protein fragments, suggesting that degradation might be from the C-terminus since the TAP tag is fused to the C-terminus.

The next step was to confirm the localization of MFP1 inside the chloroplast. First, I isolated the chloroplasts followed by isolation of MFP1 which was confirmed by SDS-PAGE and western blotting using MFP1 antibody. The bands in lane 5 of Fig. 11 show the presence of MFP1 inside the chloroplast like the finding of Jeong et al. in 2003. The multiple bands of MFP1 in Fig. 13 might be due to the processing of MFP1 during chloroplast import. The chloroplast targeting peptide is cleaved off, resulting in a ~10 kDa difference between pre-protein and processed mature protein. Three MFP1 bands might be various processing intermediates, indicating overexpression of MFP1/TAP overloading the import machinery inside the chloroplast or being less efficiently processed than wildtype protein.

According to Jeong et al. (2003), MFP1 is inside the chloroplast anchored in the thylakoid membrane with its transmembrane domain at the N-terminus and the majority of the protein with the C-terminus in the stroma. TEM micrographs of the thylakoid membranes were done to test if the TAP-tagged MFP1 had an effect on the grana stacking of the thylakoid structure between the WT, MT and transgenic Arabidopsis. As shown in Fig. 12 there was no difference observed in the stacking of the thylakoid membrane in which the MFP1 is anchored. My TEM micrograph results do coincide with Amanda Havighorst's work in 2012. If MFP1 was involved in the grana stacking of the thylakoid structure, a great loss of the stack would have been noticed but no difference between wildtype and mutant

thylakoid structure was seen. In addition, the overexpression of MFP1 does not appear to affect thylakoid structure either.

Finally, I tried to purify MFP1 along with its binding partners by optimizing TAP protocol using different detergents like digitonin, Triton X-100 and DDM in the extraction buffer. Detergents are used for solubilizing the complex while purifying it from the subcellular compartment containing it, maintaining the integrity of the complex. However, the purification with these detergents was not successful. This might be because the detergents used were not able to solubilize the intact protein complex for its purification. As an alternative method, I switched to NP-40 Alternative as detergent to test the interaction of MFP1 with the IgG beads during the first affinity purification step. As shown in Fig. 13, I was able to purify MFP1 up to its first purification step. However, multiple bands appeared which might be due to the degradation of MFP1 during the purification procedure or expression of TAP tagged MFP1 with and without the chloroplast targeting domain. When I again tried to do the complete Tandem Affinity Purification using NP-40 Alternative detergent, MFP1 got lost after the TEV elution step. I did not get any MFP1 band in the second affinity purification step (Fig. 14). TAP has two steps for protein complex purification to decrease the background noise and reduce false positive results (Van Leene et al. 2007). However, two-step purification also makes the procedure lengthy and has the risk of losing protein, which might be the reason in my case. It appeared the protein may have been lost to degradation during the TEV protease cleavage step. To counteract degradation, I used additional protease inhibitors such as E-64. E-64 was used because it is an irreversible, highly specific, cell permeable and low toxicity protease inhibitor used for *in vivo* studies. It also doesn't inhibit TEV protease, therefore it is safe to use for the TEV cleavage step of the

purification process. By using E-64 protease inhibitor along with the NP-40 Alternative detergent I was able to purify MFP1 complex (Fig. 15). Use of E-64 protease inhibitor helped to prevent the degradation of protein of interest. However, the concentration of the protein purified was not sufficient for Mass-Spectrometry (MS). The TAP protocol needs additional modifications to purify the MFP1 complex in high concentration.

Several approaches could be taken to improve the TAP procedure for chloroplast proteins. The classical TAP tag I used was designed to purify protein complexes from yeast, and it is possible that it could not function properly in plants increasing false results. A new GS tag, which combines two IgG-binding domains of protein G with a streptavidin-binding peptide, separated by two TEV cleavage sites, initially developed to study mammalian cells did increase the specificity and yield in a study of a transcription factor involved in signaling in plants done by Van Leene et al. in 2011. In 2005, Rubio et al. modified the original TAP tag to TAPa tag replacing: 1) TEV protease cleavage site with more specific and low-temperature active human rhinovirus 3C protease and 2) CBP domain with six histidine and a nine myc for purification of protein complex from a transgenic Arabidopsis. This TAPa procedure can be applied to protein expressed at different growth range, stimuli and plant materials. Construct expression depends on position effects at the integration site so to get the expressed protein of interest all the randomly inserted lines have to be tested (Dedecker et al. 2015). Dedecker et al. in 2015 explained unlike in yeast, the integration of the construct coding for protein and TAP tag takes place more efficiently in plastid and mitochondria than the nucleus by homologous recombination to make transgenic plants expressing protein of interest. Inserting the construct directly inside the chloroplast would liminate the selection

test for random integration. However, the chloroplast targeting signal in the MFP1 would have to be removed while the construct is being made.

Once the MFP1 complex can be isolated in sufficient concentration either by further improving the procedure or using an alternative tag, the identity and functions of the proteins can be studied in future experiments. Tandem Affinity Purification-Mass Spectrometry (TAP-MS) is a valuable method for purifying multiprotein complex and verifying the protein-protein interactions in the complex by mass spectrometry. However, the binary interacting protein pairs cannot be determined with the TAP-MS method. To further confirm interacting partners in a multiprotein complex and their localization in the cell, bimolecular fluorescence complementation (BiFC) assays could be done.

Literature Cited

Andrès C, Agne B, Kessler F (2011) Preparation of multiprotein complexes from Arabidopsis chloroplasts using tandem affinity purification. Methods Mol Biol 775: 31-49

Behrens C, Blume C, Senkler M, Eubel H, Peterhänsel C, Braun HP (2013) The 'protein complex proteome' of chloroplasts in *Arabidopsis thaliana*. J Proteomics 91: 73-83

Burkhard P, Stetefeld J, Strelkov SV (2001) Coiled-coils: a highly versatile protein folding motif. Trends Cell Biol 11: 82–88

Causier B, Davies B (2002) Analyzing protein-protein interactions with the yeast two-hybrid system. Plant Mol Biol 50: 855–870

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana.* Plant J 16: 735-743

Dedecker M, Van Leene J, De Jaeger G (2015) Unravelling plant molecular machineries through affinity purification coupled to mass spectrometry. Curr Opin in Plant Biol 24: 1-9

DeShields A (2017) Investigation of protein interaction partners of plant-specific coiled-coil proteins. [master's thesis]. Appalachian State University, Boone, NC

Friso G, Giacomelli L, Ytterberg AJ, Peltier JB, Rudella A, Sun O, Van Wijk KJ (2004) In-depth analysis of the thylakoid membrane proteome of *Arabidopsis thaliana*

chloroplasts: new proteins, new functions, and a plastid proteome database. The Plant Cell 16: 478-499

Gindullis F, Meier I (1999) Matrix attachment region binding protein MFP1 is localized in discrete domains at the nuclear envelope. Plant Cell 11: 1117-1128

Gindullis F, Peffer NJ, Meier I (1999) MAF1, anovel plant protein interacting with matrix attachment region binding MFP1, is located at the nuclear envelope. Plant Cell 11: 1755-1767

Godwin I, Todd G, Ford-Lloyd B, Newburg HJ (1991) The effects of acetosyringone and pH on Agrobacterium-mediated transformation vary according to plant species. Plant Cell Rep 9: 671-675

Havighorst A (2012) Analysis of the function of matrix attachment region-binding filament-like protein 1 (MFP1) in the chloroplast thylakoid membrane of *Arabidopsis thaliana*. [master's thesis]. Appalachian State University, Boone, NC

Heinemeyer J, Eubel H, Wehmhöner D, Jänsch L, Braun HP (2004) Proteomic approach to characterize the supramolecular organization of photosystems in higher plants. Phytochemistry 65: 1683-1692

Järvi S, Suorsa M, Paakkarinen V, Aro EM (2011) Optimized native gel systems for separation of thylakoid protein complexes: novel super- and mega-complexes. Biochemical Journal 439: 207–214

Jeong SY, Peffer N, Meier I (2004) Phosphorylation by protein kinase CKII modulates DNA-binding activity of a chloroplast nucleoid-associated protein. Planta 219: 298-302

Jeong SY, Rose A, Meier I (2003) MFP1 is a thylakoid-associated, nucleoid-binding protein with a coiled-coil structure. Nucleic Acids Res 31: 5175–5185

Kügler M, Jänsch L, Kruft V, Schmitz UK, Braun HP (1997) Analysis of the chloroplast protein complexes by blue-native polyacrylamide gel electrophoresis (BN-PAGE). Photosynth Res 53: 35-44

Majeran W, Friso G, Asakura Y, Qu X, Huang M, Ponnala L, Watkins KP, Barkan A, van Wijk KJ (2012) Nucleoid-enriched proteomes in developing plastids and chloroplasts from maize leaves: a new conceptual framework for nucleoid functions. Plant Physiol 158: 156-189

Majeran W, Zybailov B, Ytterberg AJ, Dunsmore J, Sun Q, van Wijk KJ (2008) Consequences of C4 differentiation for chloroplast membrane proteomes in maize mesophyll and bundle sheath cells. Mol Cell Proteomics 7: 1609-1638

Mason JM, Arndt KM (2004) Coiled-coil domains: stability, specificity, and biological implications. Chem Bio Chem 5: 170-176

Meier I, Phelan T, Gruissem W, Spiker S, Schneider D (1996) MFP1, a novel plant filament-like protein with affinity for matrix attachment region DNA. Plant Cell 8: 2105–2115

Mier P, Alanis-Lobato G, Andrade-Navarro MA (2017) Protein-protein interactions can be predicted using coiled-coil co-evolution patterns. J Theor Biol 412: 198-203

Olinares PD, Ponnala L, van Wijk KJ (2010) Megadalton complexes in the chloroplast stroma of *Arabidopsis thaliana* characterized by size exclusion chromatography, mass spectrometry, and hierarchical clustering. Mol Cell Proteomics 9: 1594-1615

Puig O, Caspary F, Rigaut G, Rutz B, Bouveret E, Bragado-Nilsson E, Wilm M, Séraphin B (2001) The tandem affinity purification (TAP) method: A general procedure of protein complex purification. Methods 24: 218-229

Rohila JS, Chen M, Cerny R, Fromm ME (2004) Improved Tandem affinity purification tag and methods for isolation of protein heterocomplexes from plants. Plant J 38: 172-181

Rohila JS, Chen M, Chen S, Chen J, Cerny R, Dardick C, Canlas P, Xu X, Gribskov M, Kanrar S, Zhu JK, Ronald P, Fromm ME (2006) Protein-protein interactions of tandem affinity purification-tagged protein kinases in rice. Plant J 46: 1-13

Rose A, Meier I (2004) Scaffolds, levers, rods and springs: diverse cellular functions of long coiled-coil proteins. Cell Mol Life Sci 61: 1996-2009

Rubio V, Shen Y, Saijo Y, Liu Y, Gusmaroli G, Dinesh Kumar SP, Deng XW (2005) An alternative tandem affinity purification strategy applied to Arabidopsis protein complex isolation. Plant J 41: 767-778

Samaniego R. Jeong SY, de la Torre C, Meier I, Moreno Díaz de la Espina S (2006a) CK2 phosphorylation weakens 90 kDa MFP1 association to the nuclear matrix in *Allium cepa*. J Exp Bot 57: 113-114

Samaniego R, Jeong SY, Meier I, Moreno Díaz de la Espina S (2006b) Dual location of MAR-binding, filament-like protein 1 in *Arabidopsis*, tobacco, and tomato. Planta 223: 1201-1206

Samaniego R, de la Torre C, Moreno Díaz de la Espina S (2008) Characterization, expression and subcellular distribution of a novel MFP1 (matrix attachment region-binding filament-like protein 1) in onion. Protoplasma 233:31-38

Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press

Van Leene J, Eeckhout D, Persiau G, Van De Slijke E, Geerinck J, Van Isterdael G, Witters E, De Jaeger G (2011) Isolation of tanscription Factor complexes from *Arabidopsis* cell suspension cultures by tandem affinity purification. Methods in Mol Biol 754: 195-218

Van Leene J, Stals H, Eeckhout D, Persiau G, Van De Slijke E, Van Isterdael G, De Clercq A, Bonnet E, Laukens K, Remmerie N, Henderickx K, De Vijlder T, Abdelkrim A, Pharazyn A, Van Onckelen H, Inzé D, Witters E, De Jaeger G (2007) A tandem affinity purification-based technology platform to study the cell cycle interactome in *Arabidopsis thaliana*. Mol Cell Proteomics 6: 1226-1238

Van Leene J, Witters E, Inzé D, De Jaeger G (2008) Boosting tandem affinity purification of plant protein complexes. Trends Plant Sci 13: 1360-1385

Veluthambi K, Krishnan M, Gould JH, Smith RH, Gelvin SB (1989) Opines stimulate induction of the vir genes of the *Agrobacterium tumefaciens* Ti plasmid. J Bacteriol 171: 3696-3703

Venkatakrishnan S, Mackey D, Meier I (2013) Functional investigation of the plant-specific long coiled-coil proteins PAMP-INDUCED COILED-COIL (PICC) and PICC-LIKE (PICL) in *Arabidopsis thaliana*. PLoS One 8: e57283

Xing S, Wallmeroth N, Berendzen KW, Grefen C (2016) Techniques for the analysis of protein-protein interactions in vivo. Plant Physiol 171: 727-758

Xu X, Song Y, Li Y, Chang J, Zhang H, An L (2010) The tandem affinity purification method: An efficient system for protein complex purification and protein interaction identification. Protein Expr Purif 72: 149-156

Zhang Y, Gao P, Yuan JS (2010) Plant protein-protein interaction network and interactome. Curr Genomics 11: 40-46

Vita

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