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Lauren Angello

Coastal Carolina University, lrangello@coastal.edu

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**Use of Co-Immunoprecipitations and 2D Gel Electrophoresis to Identify Protein-Protein
Interactions of Maturase K**

By

Lauren Angello

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Louis E. Keiner
Director of Honors
HTC Honors College

Michelle M. Barthelet, Ph

Assistant Professor
Department of Biology
Gupta College of Science

ABSTRACT

Maturase K (MatK) is the only group II intron encoded protein in the chloroplast of land plants. Maturases are prokaryotic enzymes that catalyze formation of the lariat structure needed for intron removal from precursor RNAs. The chloroplast maturase MatK, is a descendent of prokaryotic maturases, however, unlike its prokaryotic relatives, MatK is thought to catalyze excision of, not only its own intron, but also the introns of other group II introns in the plastome. Similar to the multiprotein and snRNA spliceosomal complex of the nucleus, it is postulated that MatK is not working alone to excise these introns but most likely requires additional protein factors to facilitate intron excision. In order to identify proteins that interact with MatK and understand more about splicing in the chloroplast, several laboratory methods were employed. Chloroplasts were extracted from the model organism *O. sativa japonica* and used in a co-Immunoprecipitation with anti-MatK antibody to obtain MatK and the proteins that bind to it. 2D gel electrophoresis was used to separate the proteins obtained, along with subsequent protein identification using electrospray ionization mass spectrometry.

INTRODUCTION

The appearance and evolution of chloroplasts in plant cells can be attributed to the widely accepted endosymbiotic theory. Proposed by Lynn Margulis in 1967, this theory suggests that in the primitive time of life on Earth, a photosynthetic bacterium that produces oxygen was engulfed by a larger, anaerobic eukaryotic cell (Knoll & Margulis, 2012; Alberts et al., 2002). Instead of the eukaryotic cell digesting the bacteria, it remained in the cytoplasm, carrying out photosynthesis as a symbiont (Alberts et al., 2002). This mutually beneficial relationship provided the eukaryotic cell with the ability to live as an autotroph, allowing it to exist in an anaerobic environment and the bacterium safety from the harsh conditions of the primitive world (Bobik & Burch-Smith, 2015). The photosynthetic bacterial symbiont has coevolved with the eukaryotic cell over millions of years, forming what is known today as the chloroplast of plant cells.

Along with a thick cell wall, chloroplasts are a defining feature of plant cells. The chloroplast is responsible for carrying out many metabolic functions including photosynthesis, which produces oxygen, glucose and water from carbon dioxide and light (Alberts et al., 2002). Because of its bacterial ancestry, the chloroplast has its own genome apart from the nucleus of the plant cell and is able to replicate the genetic material and divide using binary fission (Glynn et al., 2007). The plastome is circular, similar to that of its bacterial ancestor, and its expression involves the same two main processes as the nuclear genome: transcription to mRNA (messenger RNA) and translation into functional protein by a ribosome (Alberts et al., 2007).

Before an mRNA transcript (whether nuclear or plastid encoded) can be translated to its protein product, it must be modified post-transcriptionally to remove non-coding sequences called introns (Berg, Tymoczko & Stryer, 2002). The remaining exon coding regions are ligated

together in a process called splicing. In the nucleus, splicing of pre-mRNA transcripts into mature mRNA is well understood. A majority of the introns in the nuclear genome are spliceosomal introns, which require multiple proteins and small nucleolar RNAs (snRNA) that form a complex called the spliceosome in order to be excised (Will & Lührmann, 2011).

However, splicing activity is much less understood in the chloroplast.

There are three main classification for introns: spliceosomal introns of the eukaryotic nucleus, group I, and II introns. In most land plants, the chloroplast genome contains approximately twenty group II introns (Eckardt 2007). These introns can catalyze self-excision and can encode a reverse transcriptase like protein (Lambowitz & Zimmerly, 2011). It has been suggested that spliceosomal introns of the eukaryotic nucleus have evolved from group II introns (Zimmerly & Semper, 2015). When under the right conditions, the group II intron within the pre-mRNA can fold and act as a catalytic RNA (ribozyme) that can catalyze excision from the pre-mRNA (Lambowitz & Zimmerly, 2011). This is typically done with help from the intron encoded protein, such as a maturase, and/or other protein factors (Zimmerly & Semper, 2015).

Maturases have been described as an intron encoded protein that aids in specific splicing of its own intron (reviewed in Schmitz-Linneweber et al., 2015). Maturases catalyze splicing in a biochemical manner similar to that of the nuclear spliceosomal complex, excising the intron as a lariat through two transesterification reactions (Zimmerly & Semper, 2015; reviewed in Schmitz-Linneweber et al., 2015). Maturases usually have three main functional regions: an RT (reverse transcriptase) domain for intron mobility, the RNA-binding and splicing domain X and the DNA-binding/endonuclease (“zinc-finger-like”) domain (Mohr et al., 1993; reviewed in Schmitz-Linneweber et al., 2015). A similar RT domain motif is also found in Prp8, a protein in the nuclear spliceosomal complex supporting the evolutionary connection between maturases

and spliceosomal machinery (Zimmerly & Semper, 2015). Domain X, sometimes referred to as the “maturase domain,” is involved with recognition and binding to the sequence it splices, and influences formation of the ribozyme (Zimmerly & Semper, 2015). The DNA-binding/endonuclease domain allows the intron to mobilize, bind to and cut the DNA in a way that functions as a primer for reverse transcription of the intron (San Filipino & Lambowitz, 2002; Zimmerly & Semper, 2015). Even though these are the main three functional aspects of maturases, group II introns have mutations frequently (reviewed in Schmitz-Linneweber et al., 2015). This can destroy open reading frames that encode the maturase. Alternatively, parts of the domains can be lost, diminishing functional ability of the maturase.

In most land plants, the genomes of mitochondria and the chloroplast each have one maturase; MatR and MatK, respectively (reviewed in Schmitz-Linneweber et al., 2015). Maturase genes have also been found in the nucleus, likely due to transfer of genetic elements between the mitochondria and chloroplasts to the nucleus (reviewed in Schmitz-Linneweber et al., 2015).

Maturase K (MatK), encoded in the intron of the *trnK* gene, is the only maturase in the chloroplast of land plants (Nehaus & Link, 1987). It is fast-evolving due to its high rate of substitution at the nucleic acid and amino acid levels, which makes it a good candidate for plant phylogenetic analysis, even below the family level (Müller et al., 2006). MatK has lost the DNA-binding/endonuclease domain completely and lost some of the RT domain, probably due to its high mutation rate (Mohr et al, 1993). However, mutations occur typically in groups of three, preserving the open reading frame (ORF) for correct translation (Barthet & Hilu, 2008). These losses prevent MatK from being a “mobile” genetic element on its own and from other functions of typical maturases. For example, loss of portions of the RT domain that contains the RNA

recognition site may have allowed MatK to act more as a general splicing factor as opposed to typical maturases that only splice the intron they are encoded within (Vogel et al., 1999). So far, MatK has been suggested to bind and aid self-excision of seven group II intron containing transcripts in the chloroplast genome: *trnK*, *trnA*, *trnV*, *trnI*, intron two of *rps12*, *rpl2*, and *atpF* (Ems et al., 1995; Jenkins et al., 1997; Vogel et al., 1999).

MatK is likely carrying out splicing of these introns with the help of other protein factors in order to compensate for loss of functional domains. In a previous study, nuclear-encoded proteins WTF1 and RNC1 were shown to be involved in splicing the same seven chloroplast intron targets that MatK is predicted to work with (Kroeger et al., 2009). WTF1 was stated as “required” for intron splicing in the chloroplast and was detected in complex with ribonucleoprotein particles (typical of splicing activity) and the protein RNC1 (Kroeger et al., 2009). Analysis of the amino acid sequence of WTF1 (What’s This Factor 1) revealed that it is a PORR domain containing protein (Marchler-Bauer et al., 2017). The PORR domain abbreviation stand for “Plant Organelle RNA Recognition” (Marchler-Bauer et al., 2017). This type of domain contains an RNA recognition sequence specific to RNAs within the chloroplast or mitochondria, depending on which localization sequence the PORR containing protein has. MatK has lost its RNA recognition ability, making it possible that WTF1 is filling in for this lost function through protein interaction. This would also explain how MatK is able to atypically recognize and splice introns other than its own *trnK* intron. It is possible that WTF1 helps MatK recognize and excise introns other than itself as MatK has lost portions of the functional domains responsible for this action over time (Domain X). Additionally, WTF1 was found to associate with the same introns as MatK, further supporting this idea (Kroeger et al., 2009). RNC1, found in complex with WTF1, contains two ribonuclease III domains (Marchler-Bauer et al., 2017).

This type of domain is responsible for specific RNA endonuclease activity and can possibly be aiding MatK in recognition and splicing activity due to loss of the endonuclease function of the zinc finger-like domain (Marchler-Bauer et al., 2017). Like WTF1, RNC1 has also been shown to associate with the same introns as WTF1 and MatK, reinforcing the idea that these proteins are working in conjunction to carry out intron excision (Kroeger et al., 2009). However, these proteins have not yet been demonstrated as having interactions with MatK.

The purpose of my research project was to determine what proteins, if any, interacted with MatK to facilitate group II intron self-excision in the chloroplast of *Oryza sativa japonica* (rice). To do so, co-Immunoprecipitations were completed to capture MatK and the proteins it interacts with. Separation of the eluted proteins was done using 2D gel electrophoresis and the proteins pulled down with MatK can be identified from the gel using Electrospray Ionization Mass Spectrometry.

Co-Immunoprecipitations (co-IPs) are a commonly used biochemical technique to identify protein-protein interactions (Lin & Lai 2017). The basic methodology behind co-IPs involves an anti-protein of interest antibody conjugated to beads. When exposed to the total protein extract, the antibody on the beads will capture the protein of interest and any proteins that were bound to it. These proteins can then be eluted off the beads and analyzed using a variety of methods. In order to effectively use co-IPs to obtain a protein of interest and the proteins it interacts with, several considerations must be made. First, the antibody used to capture the protein of interest must be tested and validated to ensure the antibody will pull down the correct protein from a total protein sample. Additionally, thinking about when the protein of interest is expressed is also an important factor. Extracting protein from an organism without knowing when the protein of interest is expressed could result in a co-IP with little to no eluted protein.

Lastly, other techniques must be employed to separate and analyze the protein sample that was eluted off the beads.

In this study, 2D gel electrophoresis was chosen to separate the proteins within the bound sample. Bradford analysis of the bound co-IP sample provides an estimated total protein concentration that is used to determine how many nanograms of protein will be applied to the 2D gel. The basic premise behind separation of the proteins using this technique involves two main phases (or dimensions): separation by isoelectric point and by mass. Using an isoelectric focusing machine, a high voltage is applied to a gel strip loaded with a calculated amount of protein sample. The gel in the strip contains a pH gradient. When the proteins are on the strip, their net charge is dependent on where in the pH gradient they bound to. After the electric current is applied, the protein will move either toward or away from the positively charged end until the protein reaches a pH region where it no longer has a net charge and is unaffected by the current. This point is regarded as the protein's isoelectric point (pI), which is the pH at which the protein has no net charge. This point is different for every protein depending on amino acid composition. After separation by pI, the gel strip is put in contact with the well of a vertical gel for separation by mass. This second-dimension separation pulls the proteins off of the strip into the gel using a typical gel electrophoresis set up. Larger proteins move slower through the gel matrix and smaller proteins move faster when the electric current is applied. Following this second-dimension separation, gels can be stained or activated (depending on the type of gel used) to assess the pIs and masses of the protein spots obtained from the co-IP.

2D electrophoresis provides only minimal information about the proteins obtained from the co-IP. Therefore, further analysis is required to identify what each individual protein spot is. For this research, Electrospray Ionization Mass Spectrometry (ESI-MS) has been selected to

further analyze the protein spots obtained in the 2D gel. Mass spectrometry is a useful tool that can provide qualitative and quantitative data about the protein spots on a 2D gel or other biological samples (Ho et al., 2003). If the gel samples are properly prepared, mass spectrometry will output data concerning concentration, molecular mass and structure. This data is obtained by ionizing the contents of the sample using a high voltage and sending them through to the detector (Ho et al., 2003). The ions hit the detector and the signals are recorded based on the mass to charge ratio of the ions (Ho et al., 2003). There are many variations of mass spectrometry. However, electrospray ionization gives a better understanding of all of the possible charged forms of the proteins within the sample. It does so by transforming the liquid protein sample into an aerosol prior to detection by the mass spectrometer, which increases the sensitivity of the analysis and provides more accurate results about the identity of the protein spots (Ho et al., 2003).

Overall, using these techniques together may prove useful for the application of identifying protein-protein interactions with Maturase K. Understanding the protein components involved in post-transcriptional splicing in the chloroplast may help further knowledge about the evolution of maturases and splicing factors in the nucleus. It is hopeful that, using these techniques, MatK will be able to be obtained from the chloroplast along with other factors it interacts with and that they will all be able to be identified using the analysis methods employed.

METHODS

Oryza sativa japonica Growing Conditions

O. sativa japonica seeds were sterilized with 95% ethanol followed by a 50% bleach and 50% sterile water solution with subsequent washes in sterile water. Sterilized seeds were stored at 4 °C until planting. Flats and planting inserts were cleaned prior to use using bleach and deionized water and allowed to dry. Seeds were planted one centimeter deep in one third sterile *Arabidopsis* potting media including controlled release fertilizer (Lehle seeds) and two thirds commercial Vermiculite. Seeds were watered as needed. Seeds were germinated under 100% humidity at 26 °C with a 16/8-hour photoperiod. Leaf tissue was harvested after fourteen days, immediately frozen in liquid nitrogen, and stored at -80 °C until used for extraction.

Chloroplast Extraction

Chloroplast extraction was completed following a modified protocol from Qu (2015). All buffers needed were prepared fresh for each extraction. Two to three grams of tissue were placed into a Waring blender with 100 mL of grinding buffer (Final concentrations: 50mM HEPES-KOH pH 8, 330 mM sorbitol, 2 mM EDTA, 1 mM MgCl₂, 1 mM MnCl₂, 1.5 mM sodium ascorbate, 0.25% BSA, sterile water up to 200 mL). Blender was pulsed three times for five seconds each pulse. Resulting mixture was filtered through a layer of Miracloth. Samples then were centrifuged at 2,000 x g for twenty minutes at 4 °C. Supernatant was discarded and the pellet was re-suspended in 1 mL of HS buffer (Final concentrations: 50 mM HEPES-KOH pH 8, 330 mM sorbitol, sterile water up to 50 mL). Two extractions were completed at the same time with re-suspended pellets combined and an additional two milliliters of HS buffer were added. This combined fraction was then transferred to a sterile 15 mL polypropylene tube and centrifuged at 2,000 x g for twenty-five minutes at 4 °C. The supernatant was carefully removed

again, and the pellet was re-suspended in 200 microliters of EX buffer (Final concentrations: 0.2 M potassium acetate, 30 mM HEPES-KOH pH 8, 10 mM magnesium acetate, 2 mM DTT, sterile water up to 50 mL, and 0.03 μ g aprotinin). This volume was transferred into a 1.5 mL microcentrifuge tube. Extracted chloroplasts were examined under a compound microscope to determine status of intact chloroplasts prior to protein extraction. Intact chloroplasts were broken open to release protein using a sterile 24-gauge hypodermic and a syringe at 4°C by pulling sample through the needle forty times. Concentration of chloroplast protein was assayed at 595 nm of light using Bradford reagent and bovine serum albumin as the standard. Protein samples were stored at -80°C until ready for use in a co-immunoprecipitation.

Co-Immunoprecipitations

Co-Immunoprecipitations were conducted using the Pierce™ Direct Magnetic IP/Co-IP Kit following the manufacturer's protocol. Any steps not specifically stating to be completed at room temperature were done in the 4°C cold room. A 100 microliter anti-MatK antibody solution (containing 3 microliters of anti-MatK antibody (Agrisera)) was incubated with and conjugated to twenty-five microliters of magnetic beads for one hour. Unbound antibody was collected as the flow-through fraction. Beads were then washed with supplied IP Lysis/Wash buffer and quenched with the supplied Quenching buffer for one hour. Chloroplast protein ranging from 38-185 micrograms was added to IP Lysis/Wash buffer to a final volume of 500 microliters. A denatured protein sample of the same mass was prepared by incubating the protein at 95°C with fifteen microliters of β -mercaptoethanol to verify protein interactions. The denatured protein sample allows for verification of protein interactions by unfolding the protein, which will not allow any interactions to occur. Samples were incubated with the antibody-conjugated beads for two hours. Sample not bound to beads was collected as the unbound fraction. Bound protein was

eluted using the supplied Elution buffer. A Bradford assay using Coomassie and read at 595 nm was conducted to assess concentration protein within the bound fractions.

2D Gel Electrophoresis

The ReadyPrep 2D Start Kit (Bio-Rad) was used to conduct 2D gel electrophoresis on the bound sample from the co-IP. Approximately 10-20 micrograms of protein sample were added to rehydration buffer in a 1.5 mL microcentrifuge tube and allowed to incubate at room temperature for thirty minutes. A seven-centimeter IPG strip (pH 3-10) was allowed to thaw on the benchtop. The protein/rehydration buffer solution were pipetted onto the back of the channel of the rehydration tray. Plastic backing was removed from the gel side of the strip using sterile forceps and was laid gel side down into the protein/rehydration solution. This was allowed to sit for forty-five minutes and then two milliliters of mineral oil were placed in the same channel to prevent dehydration. The rehydration tray was incubated at room temperature overnight.

The isoelectric focusing tray was prepared by placing two paper wicks over the electrodes. The paper wicks were wet with 8 microliters of nanopure water. The strip was drained of mineral oil and placed gel side down (“+3-10” side toward on the left) into the focusing tray on top of the wicks. Two milliliters of mineral oil were pipetted into the tray and it was placed into the IEF machine with lid in the correct orientation. Focusing protocol suggested by the Bio-Rad protocol for a seven-centimeter strip was used at a constant 4°C. The protocol was programmed as followed; step one was set at 250 volts for 20 minutes with a linear ramp, step two was set to 4,000 volts for two hours with a linear ramp, and step three was set to 4,000 volts for 10,000 volt-hours with a rapid ramp. The last step of focusing protocol was a 99 hour hold at 100 volts until strip could be removed the following day.

Equilibration buffers to prepare the strips for SDS-PAGE had to be made fresh each time. The strip was placed gel side up into a clean rehydration tray with 2.5 mL of equilibration buffer 1 and placed on an orbital shaker for fifteen minutes (Final concentrations: 375 mM Tris-HCl pH 8.8, 6 M urea, 20% glycerol, 2% SDS, 2% DTT w/v, sterile water up to 10 mL). Equilibration buffer 1 was decanted off and 2.5 mL of equilibration buffer 2 was added to the tray and placed on an orbital shaker for fifteen minutes (Final concentrations: 375 mM Tris-HCl pH 8.8, 6 M urea, 20% glycerol, 2% SDS, 0.25 g iodoacetamide, sterile water up to 10 mL). This buffer was decanted off and the strip was dipped into SDS running buffer. The strip was inserted into the well of a 1.0 mm 10% SDS-polyacrylamide or TGX stain free gel, ensuring that the strip was in contact with the top of the gel. Overlay agarose was placed into well until full and a wick with 8 microliters of PageRuler Plus (Thermo Scientific) was inserted on the right side as a molecular weight standard for comparison. SDS-PAGE gels were run in 10X denaturing buffer at 120 volts for approximately forty-five minutes (30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of SDS in 1000 mL of sterile water). 10% SDS-polyacrylamide gels were stained with Coomassie brilliant blue R-250 and destained in a 40% methanol/10% glacial acetic acid solution prior to imaging.

A control for 2-D isoelectric focusing and protein resolution on the second-dimension gels was run using 169 micrograms of *Escherichia coli* protein sample provided in the ReadyPrep 2D Starter Kit as a positive control and resolved on TGX Stain-Free gels (BioRad). Coomassie stained and TGX activated gels were imaged using the ChemiDoc XRS+ with Image System one-minute activation setting for Stain-Free protein gels.

Preparation for ESI-MS

The In-Gel Tryptic Digestion Kit (Thermo Scientific Pierce) was used to prepare protein gel spots for analysis by electrospray ionization mass spectrometry. Five spots were selected from the 2D gel that was successful for preparation by tryptic digest and analysis by ESI-MS. Spots were excised from the gel using the tip of a sterilized glass Pasteur pipette and placed into separate 0.5 mL tubes. The gel pieces were destained, followed by incubations with reduction and alkylation buffers prepared according to the manufacturer's protocol and kit contents. After reduction and alkylation of the protein, the proteins were cleaved using Trypsin to generate smaller peptide chains for mass spectrometry analysis. Cleaved peptide chains were sent to the University of South Carolina Mass Spectrometry Center for ESI-MS protein determination.

RESULTS

Two chloroplast extractions were completed for use in two co-IPs. Before use in the co-IP, extraction samples were checked under a microscope to assess the quality of the chloroplasts obtained. Both chloroplast extractions were successful for isolated intact chloroplasts (Figure 1). Bradford analysis revealed the first co-IP using a chloroplast extraction from 2.53 grams of tissue produced a bound sample with an approximate protein concentration of 0.163 micrograms per microliter. However, no spots or protein were resolved from this sample after 2D gel electrophoresis (data not shown). The second chloroplast extraction done with 2.60 grams of tissue was used in a second co-IP, which produced a bound sample that had a protein concentration of approximately 0.321 micrograms per microliter. Using approximately twenty micrograms of protein from the second co-IP bound sample, the 2D gel that was produced showed four distinct spots of protein (Figure 2). One extra spot was taken from an unfocused area of protein. Spot number one and two were in the isoelectric range of 3.5-4 while spots three, four and five were in the 7.5-9 range. The masses of the proteins varied with spots one and two around 20 kDa and spots three, four and five around 50-60 kDa. In addition to the spots, there is a large area of unfocused protein in the same area as spots three, four and five.

Due to poor resolution of the protein product from the first co-IP, a test IEF/SDS-PAGE was performed using an *E. coli* standard provided with the ReadyPrep 2D Gel Starter Kit (BioRad). Many proteins were well resolved after 2D electrophoresis using this *E. coli* standard under the same IEF and SDS-PAGE running conditions used with the co-IP samples (Figure 3). Additionally, protein obtained in a previous co-IP was analyzed using regular SDS-PAGE and a distinct band was found around the 60 kDa mark matching the expected size of MatK (Figure 4).

DISCUSSION

As previously mentioned, MatK is the only known maturase found within the chloroplasts of most land plants (Nehaus & Link, 1987). While MatK lacks some important functional features of typical maturases, it has been shown to atypically catalyze the excision of group II introns other than its own (Vogel et al., 1999). Somehow, MatK can recognize and excise more introns even though it has lost all/some of the zinc finger-like and RT functional domains, respectively (Mohr et al,1993). Also, the same introns that MatK interacts with have been shown as targets of action by two nuclear-encoded proteins: WTF1 and RNC1 (Kroeger et al., 2009). Maybe these proteins are aiding MatK in excising not only its own group II intron but also others, where WTF1 and RNC1 (and possibly other unknown proteins) are making up for the functions MatK has lost over time. However, there has not yet been any data supporting this proposed interaction. This research intended on identifying these protein-protein interactions involving MatK.

Of the several 2D gels produced during this study, one resulted in resolved protein sufficient for analysis by ESI-MS. It cannot be determined with complete certainty what proteins were obtained in the co-IP until analyzed by ESI-MS. The co-IP trials are most certainly obtaining a protein having the same expected size as MatK, which has been shown in the past using regular SDS-PAGE (Figure 4). Previous work in our lab has demonstrated through immunoblot analysis that MatK is being pulled down through the co-IP methods being used (unpublished data). 2D gel electrophoresis separates proteins by isoelectric point and mass, making those two characteristics the only means for identification thus far post-co-IP. Spot number four was the darkest and most well-defined spot. This spot was likely Maturase K, as it was expected to have been in highest abundance in the bound sample. This was due to the fact

that MatK is what the antibody was targeting during the Co-IP. Additionally, this spot corresponds to roughly 60 kilodaltons and an isoelectric point of approximately 8.5, which were predicted as the correct molecular mass and pI for Maturase K. The identity of the other spots on this gel are unknown. It has been shown that WTF1 binds to RNC1 and other known chloroplast splicing factors, in addition to being a required factor for splicing the same introns that MatK interacts with (Kroeger et al., 2009). While there is no interaction shown between MatK and WTF1 or RNC1 in that study, it is possible that the interaction is brief and unable to be detected through the methods that were used. Perhaps RNC1, WTF1 and other unidentified proteins work in concert with MatK, making up for some of its lost functional domains. It is hypothesized that two of the spots on the 2D gel in this study may be these proteins. However, RNC1 and WTF1 are also both around 60 kilodaltons in mass and have isoelectric points around eight, which are similar to MatK. There was a large area of stained protein on the gel in the range of isoelectric points from approximately 7.5 to 9 and ranging in mass from 20 to 70 kilodaltons that was not focused into individual spots, making them unable to be analyzed by mass spectrometry (Figure 2). Perhaps the 100 V hold after the IEF protocol is not right and some settings within the focusing part of the 2D gels requires alterations to get those proteins into defined spots.

If WTF1 or RNC1 is not detected by ESI-MS, it is possible that they were not being expressed at the time point the tissue for this study was extracted. Further work will entail an expression profile analysis for these proteins to understand when they are most abundantly expressed. Extracting tissue at a different time points for use in the Co-IP trials could unveil more proteins in the splicing network involved with MatK in addition to WTF1 and RNC1.

Unfortunately, the true success of using this methodology for identifying protein-protein interactions with Maturase K cannot be fully affirmed until after the results from ESI-MS have

been considered. However, it is clear that several proteins do interact with MatK as evident by the successful 2D gel having multiple protein spots (Figure 2) and by SDS-PAGE analysis (Figure 4). Either way, more Co-IP samples must be produced to do more 2D gels, and the results obtained if successful, must be replicated as further, indisputable demonstration of interaction with Maturase K to carry out group II intron splicing in the chloroplast. Other researchers have proposed a spliceosomal-like complex of the chloroplast as well. In study focusing on *Chlamydomonas reinhardtii*, several proteins were shown to function together to facilitate excision of the group II introns from *psaA* precursor mRNAs in the chloroplast (Jacobs et al., 2013). Some of these proteins were nuclear-encoded, similar to that of WTF1 and RNC1 in this research.

CONCLUSION

Even though results from ESI-MS have not yet been received, the result of the 2D gel supports that the co-IP trials are pulling down MatK and other proteins along with it that it may interact with to carry out splicing of group II introns in the chloroplast. RNC1 and WTF1 are possible candidates; however, there are likely many more factors that interact with MatK other than just these two proteins. Optimization of the 2D gels to get better focusing in the 7.5 – 9 pI range is crucial for accurate identification of these components by ESI-MS. Hopefully, more rounds of extractions, co-IPs, 2D gels and ESI-MS can continue to be useful towards identification of protein-protein interactions of Maturase K. If these proteins can be identified, it could greatly further the understanding of chloroplast splicing activity and evolution of splicing mechanisms used by all organisms.

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FIGURES

Figure 1: Microscopic images of chloroplasts from after a chloroplast extraction. Magnification from left to right: 10X, 20X, 60X.

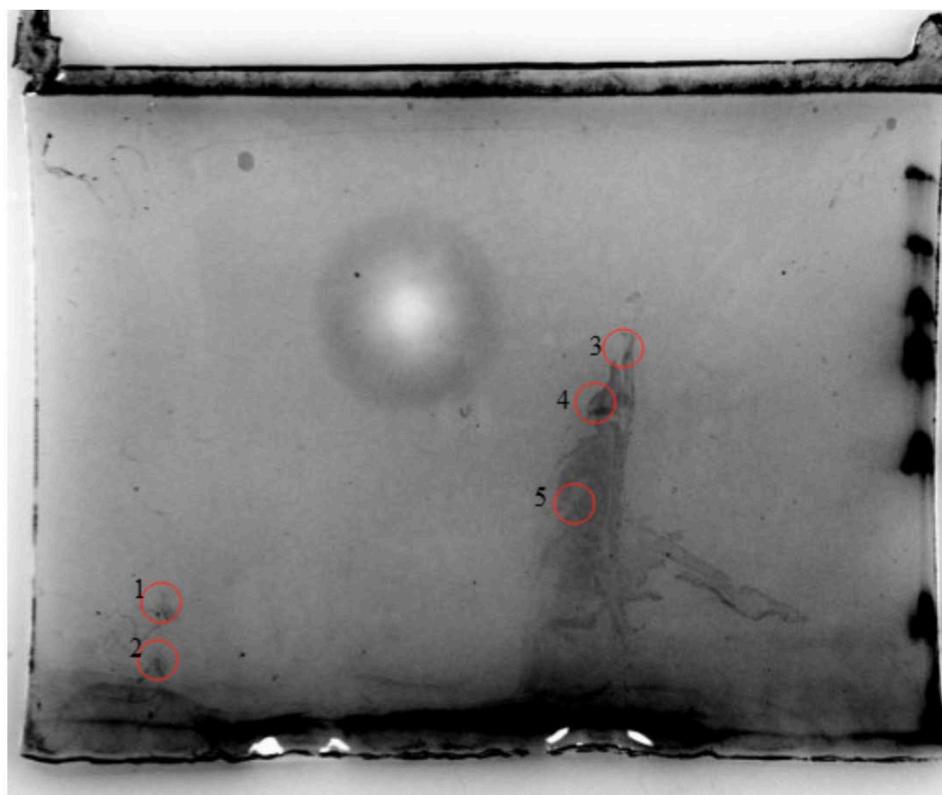


Figure 2: 2D gel result from second co-IP bound sample (0.321 g/L). Approximately 20 g of bound protein sample was loaded onto the IPG strip. Gel is 1.0 mm thick made of 10% SDS-Polyacrylamide. Gel was stained with Coomassie R-250 and destained prior to imaging with the Chemi Doc XRS+ Imaging system (Bio-Rad). PageRuler Plus Prestained protein ladder as a molecular weight standard is on the right. Spots used in ESI-MS analysis are circled and numbered.

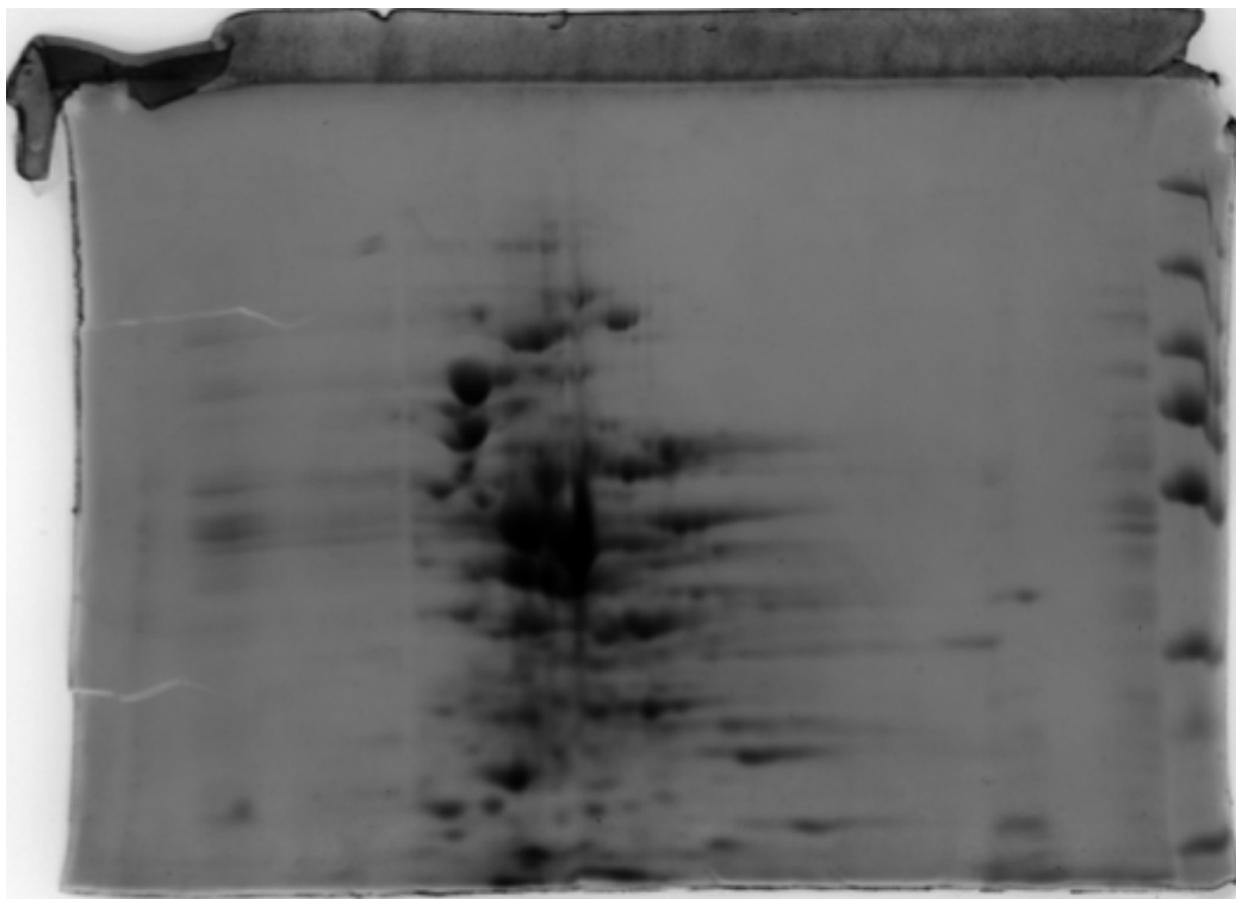


Figure 3: 2D gel result from *E. coli* positive control sample included in ReadyPrep 2D start Kit (Bio-Rad). 169 micrograms of protein was loaded onto the IPG strip. Gel is a 1.0 mm Stain-Free TGX gel that was activated prior to imaging using the Chemi Doc XRS+ Imaging system (Bio-Rad). PageRuler Plus Prestained protein ladder as a molecular weight standard is on the right.



Figure 4: SDS-PAGE of co-IP sample that used an anti-MatK antibody. Sample on the left is the bound co-IP sample and the sample on the right is the denatured bound sample. Bands correspond to 60 kDa in mass, which is the expected size of MatK.