THE GENERATION OF RECOMBINANT *Zea mays* SPASTIN AND KATANIN PROTEINS FOR IN VITRO ANALYSIS

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Plant microtubules play essential roles in cell processes such as cell division, cell elongation, and organelle organization. Microtubules are arranged in highly dynamic and ordered arrays, but unlike animal cells, plant cells lack centrosomes. Therefore, microtubule nucleation and organization are governed by microtubule-associated proteins, including a microtubule-severing protein, katanin. Mutant analysis and in vitro characterization has shown that the highly conserved katanin is needed for the organization of the microtubule arrays in Arabidopsis and rice as well as in a variety of animal models. Katanin is a protein complex that is part of the AAA+ family of ATPases. Katanin is composed of two subunits, katanin-p60, a catalytic subunit and katanin-p80, a regulatory subunit. Spastin is another MT-severing protein that was identified on the basis of its homology to katanin. In animal cells, spastin is also needed for microtubule organization, but its functionality has not yet been investigated in plants. To initiate an exploration of the function of katanin-p60 and spastin in *Zea mays*, my research goal was to generate tools for the expression and purification of maize katanin-p60 and spastin proteins in vitro. Plasmids that express katanin-p60 and spastin with N-terminal GST tags were designed and constructed via In-Fusion® cloning after traditional cloning methods were not successful. The constructs were expressed in *E. coli*, then the recombinant proteins were purified. To determine if the GST-tagged proteins are functional, ATPase activity and tubulin polymerization assays were performed. While both GST-katanin-p60 and GST-spastin hydrolyzed ATP indicating that the ATPase domains are functional, the results of the tubulin polymerization assays were less clear and further experimentation is necessary.

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INTRODUCTION

Microtubules (MTs) play essential roles in plant cell processes such as cell division, cell elongation, and organelle organization. During plant cell interphase, cortical MTs are often arranged in a parallel array and are responsible for controlling the direction of cellulose deposition and overall cell shape (Sharp et al. 2012). In dividing plant cells, MTs rearrange into dynamic and ordered arrays essential for division plane establishment, chromosome segregation, and cytokinesis (Sharp et al. 2012). *In vivo* imaging of GFP-tubulin indicates that plant cortical MTs nucleate from pre-existing MTs (Shaw et al. 2003, Murata et al. 2005). MT nucleation and organization are governed by various MT-associated proteins (MAPs), including the MT-severing protein, katanin (Stoppen-Mellet et al. 2006). MT-severing proteins generate internal breaks in MTs. Purified katanin and another MT-severing enzyme, spastin, catalyze ATP-dependent MT-severing activity *in vitro* (Hartman et al. 1998, Zhang et al. 2007).

Katanin and spastin are part of the AAA+ family of ATPases. Members of this family contribute to major biochemical pathways in the human body and act on a variety of substrates (Frickey et al. 2004). ATPases share a common feature of using the energy of ATP hydrolysis to remodel large molecular assemblies in the cell. As ATPases, katanin and spastin require ATP to sever and disassemble stable MTs (Evans et al. 2005, Salinas et al. 2005, McNally et al. 1993).

Katanin is composed of two different subunits: *p60*, a catalytic subunit, and *p80*, a regulatory subunit. The p60 catalytic subunit plays an important role in eukaryotic cell division by regulating MT length and dynamics (McNally et al. 2002). Loss of katanin-p60 function in *C. elegans* causes a failure to form a bipolar meiotic spindle (Mains et al. 1990). The catalytic subunit of katanin was identified in plants due to its sequence similarity with human katanin. In Arabidopsis, katanin severs MTs in *in vitro* and *in vivo* assays (Burk et al. 2002, Stoppin-Mellet

et al. 2002, Stoppin-Mellet et al. 2006). Arabidopsis katanin-p60 mutants have been identified in various screens and common phenotypes include a dwarfed stature, disorganized interphase cortical MT arrays, reduced cell elongation, sporadic division plane defects, and defective cellulose deposition (Burk et al. 2001). At the cellular level, release of new MTs that nucleate from existing ones is blocked in the Arabidopsis katanin-p60 mutant, which explains the lack of parallel MTs organization, defective cellulose deposition, and abnormal cell shape (Nakamura et al. 2009, Murata et al. 2005). Loss of katanin-p60 function in rice and cotton results in similar cell and plant growth phenotypes to those observed in Arabidopsis (Komorisono et al. 2005, Qu et al. 2012).

In maize, there are two katanin-p60 paralogues: *ktn2* on chromosome 3 and *clumped tassel1 (clt1)* on chromosome 8. Our lab identified a maize katanin double mutant called *discordia3 (dcd3)* that has abnormal asymmetric cell divisions and a dwarf phenotype (Amanda Wright, personal communication).

In animals, spastin was identified as a putative MT-severing protein due to its homology to katanin. Later, *in vitro* assays proved that it also severs MTs (Evans et al. 2005). Spastin is one of the most commonly mutated genes in hereditary spastic paraplegia, and it is disrupted in 40% of patients with the disease (Hazan et al. 1999). Spastin localizes to the centrosome, synaptic boutons, and points of new MT branch formation in neurons, and its overexpression induces excessive branching of MTs (Sherwood et al. 2004, Yu et al. 2008). Loss of spastin function leads to disorganized MTs arrays at the synaptic terminals of the neuromuscular junction in Drosophila (Sherwood et al. 2004, Trotta et al. 2004). In zebrafish, spastin loss leads to a disorganized axonal MTs array and impaired axonal outgrowth (Wood et al. 2006).

Unlike katanin, there are currently no published reports describing the function of any

plant spastin either *in vitro* or *in vivo*. However, searching the maize genome for genes with homology to spastin identified two spastin paralogues, one on chromosome 6 and one on chromosome 9 indicating that spastin is conserved in plants as well as animals. The maize reference inbred, B73, has an early termination stop codon in *spastin-6* which suggests that the protein encoded by *spastin-9* provides all spastin function in the maize B73 inbred line. Preliminary characterization of a putative *spastin-9* mutant suggests that loss of spastin function in maize is embryo lethal and affects endosperm development (Amanda Wright, personal communication).

The katanin and spastin ATPases have a similar protein domain structure with an N terminal MT trafficking and binding domain, followed by a linker domain, then a large C terminal ATPase domain (Fig. 1). In maize, *ktn2* and *clt1* are 97% identical at the amino acid level (Fig. 2), while Arabidopsis katanin-p60 is 81% identical to *clt1* and 80% identical to *ktn2* (Fig. 2). On the other hand, the spastin proteins in maize and Arabidopsis are only 65% identical (Fig. 3). Although maize katanin-p60 and spastin are both MT-severing proteins, only the ATPase domains are conserved as the amino acid identity between the full-length proteins is only 29% (Fig. 4).

In order to understand katanin-p60 and spastin function in maize, my research goal is to generate tools that allow for the expression and purification of maize spastin and katanin-60 proteins *in vitro*. To accomplish this, maize katanin-p60 and spastin cDNAs were cloned into an expression vector with N-terminal GST tag. The recombinant proteins were expressed in *E. coli* and column purified. To check the *in vitro* activity of the purified GST-katanin and GST-spastin, I performed ATPase activity and tubulin polymerization assays.

Figure 1. AAA ATPase protein domains.

```
Arabidopsis katanin-p60    1  MVGSSNSLAGLQDHLKLAREYALEGSYDTSVIFFDGAIAQ  40
clt1                       1     MANPLAGLQDHLKLARDYALEGLYDTSIIFFDGAIAQ  37
ktn2                       1     MANPLAGLQDHIKLARDYALECLYDTSIIFFDGAIAQ  37
                              MVGMANPLAGLQDHLKLARDYALEGLYDTSIIFFDGAIAQ

Arabidopsis katanin-p60   41  INKHLNTLDDPLARTKWMNVKKAIMEETEVVKQLDAERRA  80
clt1                      38  INKHLTTLDDALIRTKWMNCKKAISEEVEIVKQLDAQLKS  77
ktn2                      38  INKHLTTLDDALVRTKWMNCKKAISEEVESVKQLDAQLKS  77
                              INKHLTTLDDAL.RTKWMNCKKAISEEVE.VKQLDAQLKS

Arabidopsis katanin-p60   81  FKEAPTGRRAASPPINTKSSFVFQPLDEYPTSSGGGPMDD 120
clt1                      78  LKEAPGTRRSSSPPIRSNKSFVFQPLDEYPTSSP-APFDD 116
ktn2                      78  LKEAPGTRRSSSPPIRSNKSFVFQPLDEYPTSSP-APFDD 116
                              LKEAPGTRRSSSPPIRSNKSFVFQPLDEYPTSSPGAPFDD

Arabidopsis katanin-p60  121  PDVWRPPTRDVTSRRPARAGQTGTRKSPQDGAWARGPTTR 160
clt1                     117  PDVWAPP-RDTPTRRPTR-GQSSARKSSQDGAWARG-SSR 153
ktn2                     117  PDVWAPP-RDTPTRRPTR-GQSSARKSSQDGAWARG-SSR 153
                              PDVWAPPTRDTPTRRPTRAGQSSARKSSQDGAWARGPSSR

Arabidopsis katanin-p60  161  TGPASR-----GGRGGATSKSTAGARSSTAGKKGAASKSN 195
clt1                     154  TGTPSRSAKPNGIKGG-AVKS-TASNSSVR--KGKQS-SN 188
ktn2                     154  TGTPSRSSKPNGSKGGSVVKSSTASNSSVR--KGKPS-SS 190
                              TGTPSRS KPNG.KGG .VKS.TASNSSVRGKKGK SKSN

Arabidopsis katanin-p60  196  KAESMNGDAEDGKSKRGLYEGPDEDLAAMLERDVLDSTPG 235
clt1                     189  KADSTSSDAEEGKSKKGQYEGPDMDLAAMLERDVLDSTPG 228
ktn2                     191  KADSASSDAEEGKSKKGQYEGPDMDLAAMLERDVLDSTPG 230
                              KADS SSDAEEGKSKKGQYEGPDMDLAAMLERDVLDSTPG

Arabidopsis katanin-p60  236  VRWDDVAGLSEAKRLLEEAVVLPLWMPEYFQGIRRPWKGV 275
clt1                     229  VRWDDVAGLSEAKRLLEEAVVLPLWMPEYFQGIRRPWKGV 268
ktn2                     231  VRWDDVAGLSEAKRLLEEAVVLPLWMPEYFQGIRRPWKGV 270
                              VRWDDVAGLSEAKRLLEEAVVLPLWMPEYFQGIRRPWKGV

Arabidopsis katanin-p60  276  LMFGPPGTGKTLLAKAVATECGTTFFNVSSATLASKWRGE 315
clt1                     269  LMFGPPGTGKTLLAKAVATECGTTFFNVSSATLASKWRGE 308
ktn2                     271  LMFGPPGTGKTLLAKAVATECGTTFFNVSSATLASKWRGE 310
                              LMFGPPGTGKTLLAKAVATECGTTFFNVSSATLASKWRGE

Arabidopsis katanin-p60  316  SERMVRCLFDLARAYAPSTIFIDEIDSLCNSRGGSGEHES 355
clt1                     309  SERMVRCLFDLARAYAPSTIFIDEIDSLCTSRGASGEHES 348
ktn2                     311  SERMVRCLFDLARAYAPSTIFIDEIDSLCTSRGASGEHES 350
                              SERMVRCLFDLARAYAPSTIFIDEIDSLCTSRGASGEHES

Arabidopsis katanin-p60  356  SRRVKSELLVQVDGVSNTATNEDGSRKIVMVLAATNFPWD 395
clt1                     349  SRRVKSELLVQIDGVNNSSTTDDGQPKIVMVLAATNFPWD 388
ktn2                     351  SRRVKSELLVQIDGVNNSSTTEDGQPKIVMVLAATNFPWD 390
                              SRRVKSELLVQIDGVNNSSTTEDGQPKIVMVLAATNFPWD

Arabidopsis katanin-p60  396  IDEALRRRLEKRIYIPLPDFESRKALININLRTVEVASDV 435
clt1                     389  IDEALRRRLEKRIYIPLPDFESRKALININLRTVQIAADV 428
ktn2                     391  IDEALRRRLEKRIYIPLPDFESRKALININLRTVQIAADV 430
                              IDEALRRRLEKRIYIPLPDFESRKALININLRTVQIAADV

Arabidopsis katanin-p60  436  NIEDVARRTEGYSGDDLTNVCRDASMNGMRRKIAGKTRDE 475
clt1                     429  NIDEVARRTEGYSGDDLTNVCRDASMNGMRRKIAGKTRDE 468
ktn2                     431  NIDEVARRTEGYSGDDLTNVCRDASMNGMRRKIAGKTRDE 470
                              NIDEVARRTEGYSGDDLTNVCRDASMNGMRRKIAGKTRDE

Arabidopsis katanin-p60  476  IKNMSKDDISNDPVAMCDFEEAIRKVQPSVSSSDIEKHEK 515
clt1                     469  IKNMSKDEIAKDPVAMCDFVEALVKVQKSVSPSDIEKHEK 508
ktn2                     471  IKNMSKDDIAKDPVAMCDFVEALVKVQKSVSPADIEKHEK 510
                              IKNMSKDDIAKDPVAMCDFVEALVKVQKSVSPSDIEKHEK

Arabidopsis katanin-p60  516  WLSEFGSA 523
clt1                     509  WMAEFGSA 516
ktn2                     511  WMAEFGSA 518
                              WMAEFGSA
```

Figure 2. Alignment of Arabidopsis katanin-p60, maize *klt1*, and *ktn2* (dark grey shows identity, light grey shows similarity).

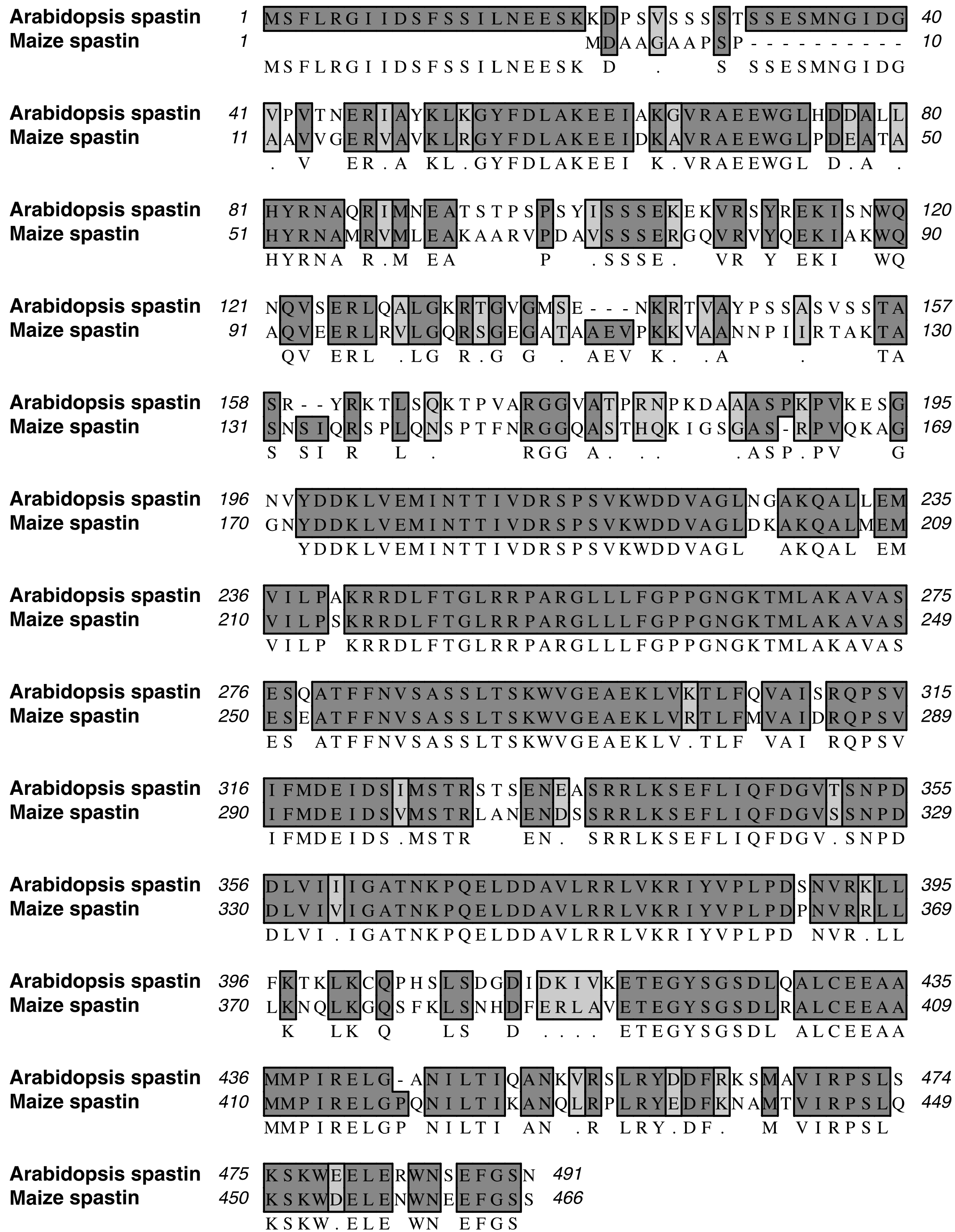

Figure 3. Alignment of Arabidopsis spastin and maize spastin (dark grey shows identity, light grey shows similarity).

```
ktn2           1   MANPLAGLQDHIKLARDYALECLYDTSIIFFDGAIAQINK   40
Maize spastin  1    MDAAGAAPSPAAVVGERVAVKLRG----YFDLAKEEIDK   35
                   M    ....    . . . . .  L   TSII.FD.A   I K

ktn2           41  HLTTLDDALVRTKWMNCKKAISEEVESVKQLDAQLKSLKE   80
Maize spastin  36  AVRAEEWGLPDEATAHYRNAMRVMLEAKAARVPDAVSSSE   75
                    .   . .L        .  A    .E         .  S E

ktn2           81  APGTRRSSSPPIRSNKSFVFQPLDEYPTSSPAPFDDPDVW   120
Maize spastin  76  RGQVR--------------------------VYQEKIAKW   89
                       RRSSSPPIRSNKSFVFQPLDEYPTSSP.  .    W

ktn2           121 APPRDTPTRRPTRGQSSARKSSQDGAWARGSSRTGTPSRS   160
Maize spastin  90  QAQVEERLR------VLGQRSGEGATAAEVPKKVAANNPI   123
                       .    RRPTRGQ  . .S   .  A .  . .

ktn2           161 SKPNGSKGGSVVKSSTASNSSVRKGKPSSSKADSASSDAE   200
Maize spastin  124 IRTAKTASNSIQRSPLQNSPTFNRGGQASTHQKIGSGASR   163
                    .   .    S. .S       .  .G   S..    .S

ktn2           201 EGKSKKGQYEGPDMDLAAMLERDVLDSTPGVRWDDVAGLS   240
Maize spastin  164 PVQKAGGNYDD---KLVEMINTTIVDRSPSVKWDDVAGLD   200
                    .     G.Y.  PDM L.  M.    ..D  .P  V.WDDVAGL

ktn2           241 EAKRLLEEAVVLPLWMPEYFQGIRRPWKGVLMFGPPGTGK   280
Maize spastin  201 KAKQALMEMVILPSKRRDLFTGLRRPARGLLLFGPPGNGK   240
                    AK .L E V.LP     . F G.RRP .G.L FGPPG GK

ktn2           281 TLLAKAVATECGTTFFNVSSATLASKWRGESERMVRCLFD   320
Maize spastin  241 TMLAKAVASESEATFFNVSASSLTSKWVGEAEKLVRTLFM   280
                   T LAKAVA.E   TFFNVS  .L SKW GE E. VR LF

ktn2           321 LARAYAPSTIFIDEIDSLCTSRGASGEHESSRRVKSELLV   360
Maize spastin  281 VAIDRQPSVIFMDEIDSVMSTRLAN-ENDSSRRLKSEFLI   319
                   .A    PS IF DEIDS....R.A GE .SSRR.KSE L.

ktn2           361 QIDGVNNSSTTEDGQPKIVMVLAATNFPWDIDEALRRRLE   400
Maize spastin  320 QFDGVSSNPDD------LVIVIGATNKPQELDDAVLRRLV   353
                   Q DGV      EDGQPK.V V..ATN P ..D.A. RRL

ktn2           401 KRIYIPLPDFESRKALININLR-TVQIAADVNIDEVARRT   439
Maize spastin  354 KRIYVPLPDPNVRRLLLKNQLKGQSFKLSNHDFERLAVET   393
                   KRIY.PLPD   R..L.  .L.G     .      . .A  T

ktn2           440 EGYSGDDLTNVCRDASMNGMRRKIAGKTRDEIKNMSKDDI   479
Maize spastin  394 EGYSGSDLRALCEEAAMMPIRELGPQNILTIKANQLR---   430
                   EGYSG DL   .C  .A M   R  .          N  .DDI

ktn2           480 AKDPVAMCDFVEALVKVQKSVSPADIEKHEKWMAEFGSA   518
Maize spastin  431 ---PLRYEDFKNAMTVIRPSLQKSKWDELENWNEEFGSS   466
                   AKDP.   DF  A   .  S.     .  E W  EFGS
```

Figure 4. Alignment of *ktn2* maize spastin (dark grey show identity, light grey shows similarity).

MATERIALS AND METHODS

RNA Extraction

The basal 2-3.5 cm of all immature leaves from a single *Zea mays* (B73) plant were collected, frozen in liquid nitrogen, and ground to a fine powder using a mortar and pestle. Next, 700 µl TRIzol was added and the homogenized sample was incubated for 5 minutes at room temperature. 160 µl chloroform was added and the samples were shaken vigorously for 15 seconds and then incubated for 3 minutes at 4°C. The sample was then centrifuged for 15 minutes at 12,000 x g at 4°C. After centrifugation, the top aqueous phase was collected and placed in a new tube. Next, 350 µl of isopropanol was added and the sample was incubated at room temperature for 10 minutes then centrifuged at 12,000 x g for 10 minutes at 4°C to pellet the nucleic acids. The pellets were washed with ethanol and centrifuged for 5 minutes then air dried. Lastly, the pellets were resuspended in 50 µl RNase-free water and incubated in water bath at 55°C for 10 minutes to promote resuspension. RNA concentrations were determined by measuring the absorbance by Nanodrop and samples were stored in -70°C.

cDNA Synthesis

Maize cDNA was synthesized using the RETROscript kit (ThermoFisher Scientific Company™) and by following their standard protocol. Briefly,2 µg of total maize B73 RNA, 2 µl of dT primers and 8 µl of ddH_2O were incubated at 85°C for 3 min then placed on ice for 30 seconds. Next, 2 µl of 10x RT buffer, 4 µl of dNTP mix, 1 µl of RNase inhibitor, and 1 uL of MMLV-RT were added and the reaction was incubated at 44°C for 1 hour and then at 92°C for 10 minutes. cDNA was stored at -20°C.

Cloning of Katanin and Spastin cDNAs into pGEM-T Easy

Katanin-p60 and Spastin cDNA Amplification

Maize katanin-p60 and spastin cDNAs were amplified using polymerase chain reaction (PCR). The reactions contained 2 µl 10x Ex-Taq buffer, 1.6 µl 2.5 mM dNTPs, 1 µl DMSO, 0.1 µl Ex Taq, 11.3 µl ddH_2O, 1 µl 100ng/µl forward primer, 1 µl 100ng/µl reverse primer and 1 µl cDNA template. For the katanin-p60 amplification the 3kat_ Psh_for and 3kat_ Psh_rev primers were used while the Spas9_ Psh_for and Spas9_ Psh_rev primers were used to amplify spastin. For primer sequences, see Table 2. The thermal cycler profile cycled through the following temperatures and incubation times: denaturation at 94°C for 2 minutes, annealing at 68°C for 1 minute, and extension at 72°C for 1 minute. This profile was repeated for 35 cycles with a final elongation for 10 minutes at 72°C.

Agarose Gel Electrophoresis and Gel Purification of PCR Products

PCR products were combined with 6x loading dye, loaded into 0.8% TAE agarose gel, and run at 110 V for 80 min. The gel was stained with 1x Sybr safe for 20 min while shaking. After confirming that the amplicons were the expected size, DNA bands were excised from the agarose gel using a clean razor blade and then placed into 1.5 mL microcentrifuge tubes. The PCR fragments were purified using Gel DNA recovery kit (Zymo Research™). Briefly, 3 ADB buffer volumes of the agarose block's weight was added to the microcentrifuge tubes. The samples were incubated at 55°C for 10 min until the agarose was totally dissolved. The solution was transferred to a Zymospin column and centrifuged for 60 sec. The spin columns were washed with 200 µl of Wash Buffer then centrifuged for 30 seconds two separate times. After the

final wash, the spin columns were transferred to clean 1.5 mL micro-centrifuge tubes and 12 µl ddH_2O was added. A 30 second centrifugation eluted the DNA into the water.

Ligation of Katanin-p60 and Spastin into pGEM Easy T Vector

Purified katanin-p60 and spastin PCR products ligated into the pGEM-T easy vector (Promega™). Each ligation reaction contained 5 µl 2X Rapid Ligation Buffer, 1 µl pGEM-T Easy vector, 3 µl of PCR product, and 1 µl T4 DNA Ligase. Samples were incubated at 4°C overnight.

Transformation of Ligations into E. coli

High Efficiency NEB 5-alpha Competent *E. coli* (New England Biolabs™), were thawed in ice for 10 minutes, and then 2 µl of each ligation reaction and 50 µl of cells were combined in a sterile 1.5ml microcentrifuge tube. Reactions were placed in ice for exactly 30 min. Then, the cells were heat-shocked for 30 seconds in a 42°C water bath. The tubes were returned to ice for 5 minutes, 950 µl room-temperature SOC medium was added to all tubes, and they were incubated for 1 hour at 37°C while shaking (~250rpm). 100 µl of each transformation culture was plated onto duplicate LB/ampicillin/IPTG/X-Gal plates and incubated for overnight (16–24 hours) at 37°C. Colonies that contain pGEM-T vectors with either spastin or katanin-p60 inserted remained white, while blue colonies contain only empty vector.

Plasmid DNA Isolation

Single white colonies were picked into culture tubes containing 5 ml LB/ ampicillin broth, then incubated at 37°C for 16 hours (overnight) while shaking at ~250 rpm. Plasmid DNA

was isolated from *E. coli* cultures using the Zyppy plasmid miniprep kit (Zymo Research™). Briefly, 3 ml of bacterial culture was pelleted in a 1.5 ml microcentrifuge tube by centrifugation. 200 ml of 7X Lysis Buffer was added to the cells and the pellet was disrupted by vortexing. 200 µl of 7X Lysis Buffer and 400 µl of cold Neutralization Buffer were added and the sample was mixed by inversion. Next, samples were centrifuged at 12000 rpm for 2 min then the supernatant was transferred a spin column. After a 30 second centrifugation, the flow-though was discarded and 200 µl Wash Buffer was added. After a 30 second centrifugation, the spin column was transferred to clean microcentrifuge tube and 30 µl ddH_2O was added to the column. A 30 second centrifugation eluted the plasmid DNA and the DNA samples were stored at -20°C.

Restriction Digests to Confirm pGEM Insert Identity

Katanin-p60 and spastin pGEM plasmids were digested with different restriction enzymes (NEB™) in a reaction containing 18 µl ddH_2O, 2.5 10x Cut Smart buffer, 0.5 µl (restriction enzyme) , and 4 µl of plasmid. The reactions were incubated overnight at 37°C, then electrophoresed on a 0.8% TAE agarose gel. Samples that gave the expected fragment sizes were sent for sequencing by Genewiz™ Using primers T7 and SP6 to confirm the presence of the correct insert and to identify mutation-free clones.

Cloning Katanin-p60 and Spastin into the pET42a Vector

Restriction Digests

After restriction digest with *PshA1*, katanin-p60 and spastin inserts were excited from pGEM-T easy vectors and pET42a were linearized with same restriction enzyme as well. Next, gel purification was followed as previously described.

Ligation, Transformation, and Evaluation of Resulting Clones

Five µl of instant Sticky-end Ligase Master Mix (NEB™) and a 3:1 molar ratio of digested katanin-p60 or spastin and 2 µl linearized pET42a vector were combined. Then, 2 µl immediately transformed into High Efficiency NEB 5-alpha Competent *E. coli* (New England Biolabs™) as previously described. About 50 single colonies were picked and grown in 5 ml LB/kan broth while shaking at 37°C overnight. Then, the plasmids were miniprepped as previously described. Plasmids were digested with *HpaI* restriction enzyme and electrophoresed on a 0.8% TAE agarose gel. Plasmids that showed the expected digestion pattern were sequenced by GeneWiz™ using primers 5GEX and T7-Term to determine if the plasmid contained the correct insert and if the insert was mutation-free.

In-Fusion Cloning of the Katanin-p60 and Spastin cDNA into pET42a Vector

Amplification of the Katanin-p60 and Spastin cDNAs

Primers were designed using Takara Bio Company's online primer design tool. PCR reactions containing 12.5 µl of CloneAmp HiFi PCR Premix from In-Fusion® HD Cloning Kit, 2 µl of each 100 ng/ µl primer, 2 µl of maize cDNA, and 8.5 µl ddH_2O. The katanin-p60 specific primers were IF_3katfor and IF3katrev and the spastin specific primers were spastin_IF_rev and spastin_IF_for. The thermal cycle profile temperature and incubation times were as follows: denaturation at 98°C for 10 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 15 sec. The thermal cycle profile was repeated for 30 cycles. PCR products were analyzed by electrophoresis on 0.8% TAE agarose gel subsequently gel purified as described previously.

In-Fusion Reactions Inserting Katanin-p60 and Spastin into pET42a

The pET42a vector was linearized by digestion with *PshA1*, electrophoresed on 0.8% TAE agarose gel, then gel purified. 2 µl of linearized pET42a vector (200 ng) and 4 µl of purified katanin-p60 and spastin PCR fragments (50 ng) were mixed with 2 µl of 5x In-Fusion HD Enzyme Premix. The reactions were incubated at 50°C for 15 min, then placed on ice. Transformations of the in-fusion reactions were as described previously. pET42a vectors containing katanin-p60 or spastin inserts were identified via restriction digest with *HpaI* for katanin-p60 and *NcoI* for spastin. Constructs containing inserts were sequenced by GeneWiz™ using primer 5GEX (GeneWiz Legacy Primer) and T7-Term (GeneWiz Universal Primer) to identify mutation free clones.

Transformation of pET42a + Katanin-p60 and pET42a + Spastin to BL21 (DE3) E. coli

The pET42a + katanin-p60 and pET42a + spastin constructs were transformed as previously described into BL21 (DE3) competent cells (NEB) for protein expression.

Small Scale Protein Expression and Induction

Single colonies from the katanin-p60 and spastin BL21 (DE3) transformations were cultured in 5 ml LB/Kan at 37°C (while shaking at 250 rpm) for 2-3 hours till the OD concentration of cells at 600 nm (as measured by Nanodrop) was between OD 0.5 to OD 1.0. Next, the entire culture was added to an autoclaved flask containing 100 ml LB/Kan. This culture was shaken at 37°C (while shaking at 250 rpm) for another 2-3 hours till the OD concentration of the cells was between OD 0.5 to OD 0.8. After reaching the desired concentration, the culture was equally split into two autoclaved flasks. 200 µl 100 mM IPTG was added to one flask to

induce expression of pET42a vector, while the other was maintained as the uninduced control. The two flasks were incubated at 37°C while shaking at 250 rpm for an additional 4 hours. Two 1 ml aliquots from each culture were removed each hour; the first one was used for measuring the concentration and the other one was centrifuged for 1 min and the pellet preserved. The remaining 42 ml of culture were centrifuged at 20,000 rcf for 20 min, the supernatant was discarded and the pellet was stored at -80°C.

Different incubation temperatures, incubation lengths and concentrations of IPTG were tried to find the optimal environment for katanin-p60 and spastin protein expression. As a result, inducing with 0.4 mM IPTG for 48 hours at 16°C was optimal for katanin-p60 expression while inducing at 0.1 mM IPTG for 48 h at 14°C was optimal for spastin.

Protease Inhibitor

Protease inhibitors cocktail (Calbiochem™) was reconstituted with 1 ml DMSO and 4 ml water and stored at -20 °C. 125 µl was added to each 0.5 g *E. coli* cells.

SDS-Page

The small scale-bacterial pellets were resuspended in 160 µl 1xPBS till the pellet completely dissolved. Next, 20 µl 4x SDS Buffer and 20 µl of 1Mwas added. The mixture was passed through a 27 µm needle several times to break open cells, then the tubes were incubated in 85°C for 3 min to denature the proteins.

20 µl of each sample and 5 µl Protein Standard Ladder (BioRad) were loaded into 12% SDS-PAGE gels. The SDS-PAGE gels were run in 1x SDS-PAGE running buffer for 30 min at 70 V and then 40 min at 180 V.

Coomassie Blue Staining

An SDS-PAGE gel was fixed in 50% methanol and 10% acetic acid. Next, the gel was stained with Coomassie Blue Buffer for 2 hours. Subsequently, gel was destained in 5% methanol and 7.5% acetic acid until the background is clear (2-4 hours).

Western Blotting

Prior to Western blotting, a SDS-PAGE gel was soaked in Transfer Buffer for 15 min. One piece of Immobilon-P membrane (BioRad™) was cut and labeled then soaked in methanol for 5 min, rinsed in ddH_2O until no beading was visible, soaked in ddH_2O for 5 min then placed in Transfer Buffer for 5 min. A transfer sandwich was created by layering the following items from the white top to the black bottom on a BioRad gel transfer cassette: wet fiber sponge, wet Whatman paper, membrane, gel (bottom was at top, ladder on the left), wet Whatman paper, wet fiber sponge. All items were wet with transfer buffer. The transfer sandwich along with an ice block was placed into the Bio-Rid electrophoresis rig filled with transfer buffer. A 100-mV current was applied for 1 hour to move the proteins from the gel to the membrane. After transfer was complete, the membrane was unpacked and air dried.

Immunodetection

A membrane containing transferred protein was hydrated in methanol for 5 min, rinsed with 1x TBS 3 times, and then washed in 1x TBS for 5 min. To block the membrane, it was incubated while shaking in 50 mg/ml BSA in 1x TBS for 1 hour. The blot was incubated with 1 µl GST primary antibody (Thermo Fisher Scientific™) which was diluted in 1 ml 1mg/ml BSA in 1x TBS at 4°C overnight. After incubation, the blot was rinsed and washed in 1x TBS with

0.05% tween for 10 min 3 times. Next the blot was incubated with 1 µl Mouse IgG Binding protein secondary antibody (Santa Cruz Biotechnology) diluted in 2.5ml 1mg/ml BSA in 1x TBS at room temperature while shaking for 1.5 hour. The blot was rinsed and then washed in 1x TBS with 0.05% tween for 10 min. Finally, the membrane was washed in alkaline phosphatase buffer for 10 min then incubated in 10 ml of alkaline phosphatase buffer with 66 µl 50 mg/ml NBT and 33 µl 50 mg/ml BCIP for 10 min. After development of the purple color signifying the location of the immunodetected proteins, the blot was rinsed 3 times in cold water.

Silver Staining

To detect small amounts of protein on SDS-PAGE gels, a silver stain kit (ThermoFisher Scientific™) was used. After electrophoresis, a gel was washed with ultrapure water two times for 5 minutes. Then the gel was fixed in 30% ethanol and 10% acetic acid two times for 15 minutes. After that, the gel was washed twice in a 10% ethanol solution for 5 minutes and twice in ultrapure water for 5 minutes. The gel was incubated in Sensitizer Working Solution for exactly 1 minute, then washed with two changes of ultrapure water for 1 minute each. The gel was incubated in Stain Working Solution for 30 minutes, then washed quickly with two changes of ultrapure water for 20 seconds each. Developer Working Solution was added immediately until protein bands appeared. When the desired band intensity was reached, developer Working Solution was replaced with prepared Stop Solution (5% acetic acid). The gel was washed briefly in Stop Solution and incubated in fresh Stop Solution for 10 minutes.

Purification of GST-Tagged Katanin-p60 and Spastin Proteins

3 ml of BugBuster Master Mix reagent (Sigma™) was added to frozen bacterial pellets obtained from 50 ml cultures. The cell suspensions were incubated on a shaking platform for 20 min at room temperature. The suspensions were then centrifuged at 4°C for 20 min at 20,000 g to remove insoluble cell debris. The supernatant containing the bacterial proteins was transferred into a new sterile tube.

The supernatant was run over a GSTrap FF Column (GE Healthcare™) which contains Glutathione Sepharose 4. GST-tagged proteins bind to the Glutathione Sepharose and other proteins move through the column. First, the column was equilibrated with 5 column volumes of binding buffer (PBS, pH 7.3). Next, bacterial proteins extracts were injected into GSTrap FF Column by syringe at a rate of 1 ml/min. Then, the column was washed with 10 volumes of binding buffer. Lastly, the elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) was applied to elute GST-katanin-p60 or GST-spastin.

Samples were exchanged with PBS buffer by using Amicon Ultra-15 Centrifugal Filter Units to remove glutathione from the eluted protein.

Small Scale Optimization of GST Tag Cleavage

Factor X was used to cleave the GST tag from the GST-katanin-p60 proteins. Initial attempts at a small scale cleavage used Factor X to katanin-p60 ratios of 1:10, 1:20, and 1:50. First, three dilutions of Factor Xa in Xa Dilution/Storage Buffer produced solutions having 1.0, 0.5, and 0.2 µg enzyme per µl. Next, 5µl of 10X Factor Xa Cleavage/Capture Buffer, 10 µg target protein, 1µl of each concentration of diluted Factor Xa and up to 50 µl to deionized water were combined and incubated at room temperature (25°C). 10 µl samples were removed after 2,

4, 8, and 16h, 10 µl 2X SDS sample buffer was added, and the samples were stored at –20°C until SDS-PAGE analysis. The extent of cleavage of the GST from the samples were determined by SDS-PAGE analysis.

ATPase Activity Assay

Reagent Preparation

ATPase activity tests were performed using KINESIN ATPase END-POINT BIOCHEM KIT (Cytoskeleton™). First, the MTs included in the kit were reconstituted by aliquoting 500 µl of Kinesin Reaction Buffer into an microcentrifuge tube and warming it at 37°C for 10 minutes. Next, the Kinesin Reaction Buffer was supplemented with 5 µl Taxol stock and 100 µl of this buffer was added to each provided tube of MTs. After 10 minutes of incubation, the MTs were pooled together and 40 x 10 µl aliquots were snap frozen in liquid nitrogen and stored at -70°C. The taxol was reconstituted with 100 µl of DMSO and was stored at -70 °C. The Kinesin Motor Control Protein was reconstituted in 10 µl of Kinesin Reaction Buffer to give a 5 mg/ml stock then aliquoted into 10 x 2 µl volumes, snap frozen in liquid nitrogen and stored at -70°C. 55.1 mg of ATP disodium salt (Sigma) was resuspended in 900 µl of 100 mM PIPES buffer pH 7.0. The pH of the solution was adjusted to pH 7.0 with 5 M NaOH and the final volume was added to 1 ml. Solution was aliquoted into 50 x 20 µl volumes and store these at -20°C.

ATPase Activity Assay

First, 1 ml of Kinesin Reaction Buffer was aliquoted into a microcentrifuge tube at room temperature. Next, one tube of taxol was thawed and added reaction buffer. After that, one 20 µl aliquot of ATP stock was thawed and diluted with 1 ml of ice cold water and placed on ice. Also,

one tube of MTs were defrosted by placing in a room temperature water bath for 2-3 minutes. Next, 240 µl of taxol supplemented reaction buffer was added to the MTs to produce a working stock of 0.2 mg/ml. After that, one tube of kinesin was thawed and diluted with 58 µl of Reaction Buffer to give a working concentration of 0.08 µg/ul. Next, the reactions were assembled in a 96 well plate as described in Table 1.

Table 1. Reaction composition for ATPase activity reactions

Reaction Buffer Plus Taxol (µl)	**Microtubules (0.2 mg/ml) (µl)**	**Kinesin heavy chain Control Protein (0.08 mg/ml) (µl)**	**Katanin-p60 or Spastin**	**Well Designation**
30	---------	---------	---------	Blank
20	10	---------	---------	MT only Control
20	10	---------	---------	MT only Control
17.5	10	2.5	---------	KHC ATPase #1
17.5	10	2.5	---------	KHC ATPase #2
17.5	10	2.5	---------	KHC ATPase #3
To 30 µl final volume	---------	---------	0.2 µg	Katanin or spastin control #1
To 30 µl final volume	---------	---------	0.4 µg	Katanin-p60 or spastin control #2
To 30 µl final volume	---------	---------	0.6 µg	Katanin-p60 or spastin control #3
To 30 µl final volume	---------	---------	0.8 µg	Katanin-p60 or spastin control #4
To 30 µl final volume	---------	---------	1.0 µg	Katanin-p60 or spastin control #5
To 30 µl final volume	10	---------	0.2 µg	Katanin-p60 or spastin test #1A
To 30 µl final volume	10	---------	0.2 µg	Katanin-p60 or spastin test #1B
To 30 µl final volume	10	---------	0.4 µg	Katanin-p60 or spastin test #2A
To 30 µl final volume	10	---------	0.4 µg	Katanin-p60 or spastin test #2B
To 30 µl final volume	10	---------	0.6 µg	Katanin-p60 or spastin test #3A
To 30 µl final volume	10	---------	0.6 µg	Katanin-p60 or spastin test #3B
To 30 µl final volume	10	---------	0.8 µg	Katanin-p60 or spastin test #4A

Reaction Buffer Plus Taxol (µl)	Microtubules (0.2 mg/ml) (µl)	Kinesin heavy chain Control Protein (0.08 mg/ml) (µl)	Katanin-p60 or Spastin	Well Designation
To 30 µl final volume	10	---------	0.8 µg	Katanin-p60 or spastin test #4B
To 30 µl final volume	10	---------	1.0 µg	Katanin-p60 or spastin test #5A
To 30 µl final volume	10	---------	1.0 µg	Katanin-p60 or spastin test #5B
To 30 µl final volume	10	---------	1.0 µg	Katanin-p60 or spastin test #5C

To initiate the ATPase assay, 5 µl of ATP stock was added to each reaction giving a 0.3 mM final concentration. Reactions were then allowed to proceed at room temperature for exactly 5 minutes. The reactions were terminated by adding 70 µl of CytoPhos to each well. After a 10 min incubation, the reactions were evaluated using a spectrophotometer (The Synergy Mx BioTek's) set to read an end-point assay with an absorbance of 650 nm.

Tubulin Polymerization Assay

Reagent Preparation

Tubulin polymerization tests were performed using Tubulin Polymerization Assay Kit (Cytoskeleton™). First, the general tubulin buffer included in the kit was reconstituted with 10 ml of sterile distilled water. Next, tubulin protein was reconstituting by mixing 1.1 ml of ice cold general tubulin buffer with 10 µl of 100 mM GTP and supplement the mixture to tubulin powder. After completely resuspended, the tubulin protein was aliquoted into 5 x 200 µl and snap frozen in liquid nitrogen and stored at -70 °C. GTP was reconstituted with 100 µl of sterile distilled water and aliquoted into 10 x 10 µl then frozen at -70°C. Paclitaxel was reconstituted with 100 µl DMSO and frozen at -70°C. Cold (4°C) Tubulin Polymerization (TP) buffer was made by

mixing 750 µl General Tubulin Buffer, 250 µl Tubulin Glycerol Buffer, and 10 µl GTP Stock (100 mM).

Spectophotometer Set Up

First Spectrophotometer (The Synergy Mx BioTek's™) Parameter Setting was prepared as follows: Kinetic Measurement type 61 cycles of 1 reading per minute. The Absorbance wavelength was 340 nm, and the Temperature 37°C and Shaking 5 s medium, orbital.

Tubulin Polymerization Assay

10 µl of General Tubulin Buffer was pipetted into two control wells (tubulin minus compound controls). GST-katanin-p60 and spastin were added at total volume of 10 µl with concentration of 0.2 µg, 0.5 µg, and 1.0 µg. 10 µl $CaCl_2$ was added at concentration 10 mM. 10 µl of the Paclitaxel Stock solution was diluted with 190 µl of room temperature General Tubulin Buffer and 10 µl was used per well (10 µM final). 200 µl of tubulin was diluted with 420 µl of ice cold TP buffer and pipette 100 µl of the diluted protein into each well. Immediately, the samples were place in reader at 37°C and start recording using the kinetic set-up described above.

RESULTS

As my primary goal was to express and purify recombinant katanin and spastin proteins for *in vitro* analysis, I chose to tag the proteins with GST to facilitate protein purification and to express the recombinant proteins in *E. coli*. To create these tagged proteins, the maize katanin-p60 and spastin cDNAs were cloned in frame with the GST ORF of the pET42a vector (Fig. 5).

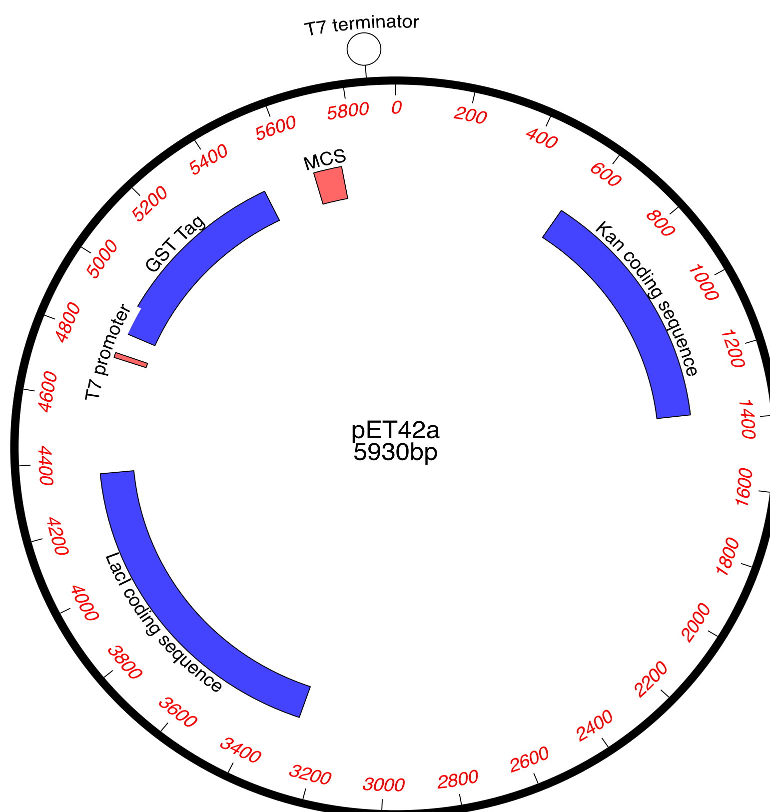

Figure 5. Schematic of the pET42a vector containing a GST tag adjacent to a multiple cloning site (MCS). pET42a size is 5930 bp.

The key features of the pET42a vector include a T7 promotor that allows for high level IPTG-induction of target protein expression, a lacI coding sequence that represses transcription of the T7 RNA polymerase gene, a kanamycin selection marker, a gene encoding the GST-tag and a multiple cloning site (MCS) at the 3’end of the GST ORF. Also, pET42a has His tag and S

tag I cloned the katanin-p60 and spastin ORFs in-frame with the GST ORF so upon induction recombinant proteins with an N terminal GST tag would be expressed.

Glutathione S-transferase (GST), which is 220 amino acids, was chosen because it can be stably and efficiently ligated with a protein of interest, and it can be detected and purified easily. Most importantly, the GST tag enhances the solubility of the tagged protein.

Since katanin p-60 is the catalytic subunit that performs the severing activity (Burk et al. 2002. Stoppin-Mellet et al. 2002, Stoppin-Mellet et al. 2006), *ktn2* was chosen to produce the recombinant protein. Spastin-9 was chosen since spastin-6 has an early termination stop codon.

Cloning of Katanin-p60 and Spastin into pET42a by Restriction Ligation

The first approach I employed to generate the needed constructs was to insert katanin-p60 and spastin into pET42a using traditional cloning methods based on restriction digestion and ligation. The cDNAs of katanin-p60 and spastin were amplified via polymerase chain reaction (PCR; Fig. 6), ligated into the pGEM T easy vector, then transformed to *E. coli*. The reason for cloning katanin-p60 and spastin into pGEM T easy vector is to avoid amplifying PCR products every time I try to clone genes of interest into the pET42a.

Transformed *E. coli* were screened for the presence of the vector containing an insert using restriction digest (Fig. 7). Plasmid DNA isolated from successfully transformed bacteria was sequenced. Mutation free katanin and spastin inserts were cut out of the pGEM vector with restriction enzymes, gel purified, then ligated into the linearized pET42a empty vector (Fig. 8).

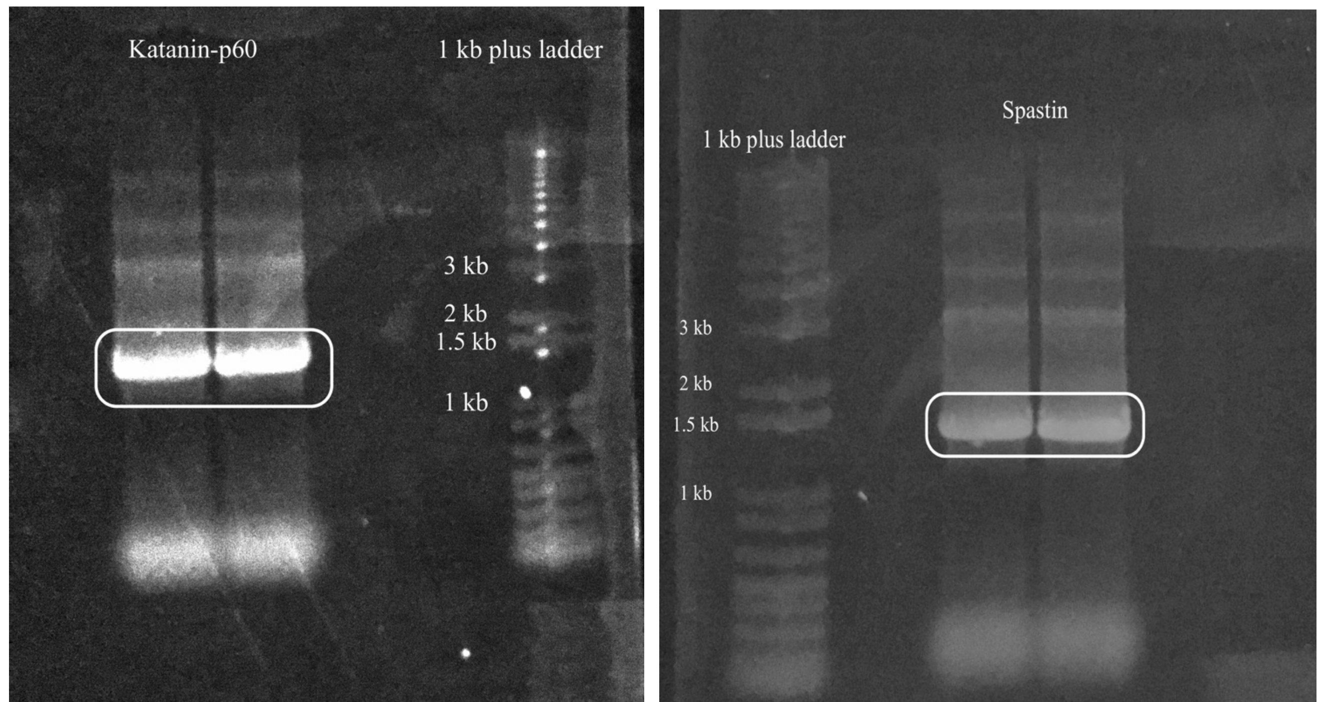

Figure 6. PCR amplified katanin-p60 and spastin from *Zea mays* cDNA. The expected band sizes are 1.6 kb for katanin and 1.5 kb for spastin.

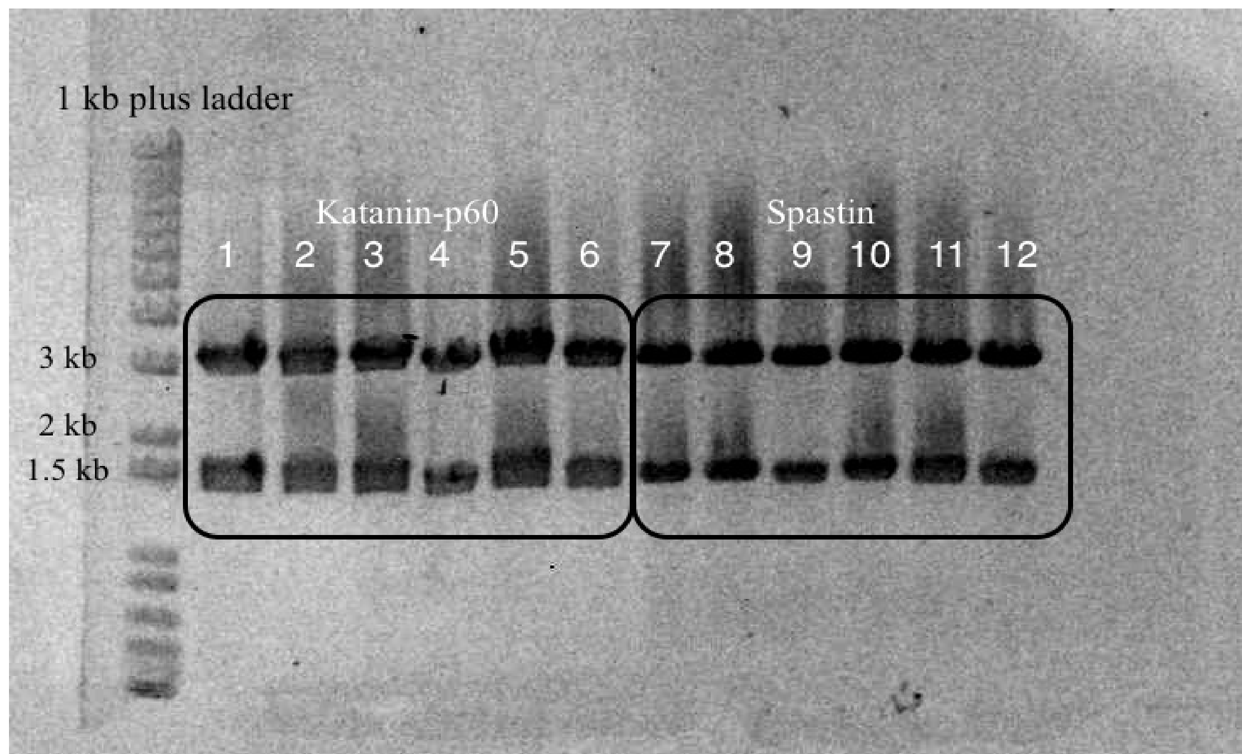

Figure 7. Restriction digest of pGEM vectors containing katanin or spastin. The expected band sizes are 3 kb (vector) and 1.5 or 1.6 kb (insert).

Figure 8. Linearized pET42a vector after restriction digestion with PshAI.

Next, I transformed *E. coli* cells with the pET42a vector plus katanin-p60 ligation. Transformed colonies were miniprepped and the integrity of the constructs were tested by restriction digest (Fig. 9) and sequencing. The results were not in accordance with my expectations, since most of the restriction digests showed randomly sized fragments on the gel (Fig. 9). The few samples that appeared to be of the expected size were sent for sequencing but a mutation free sample could not be identified. After completely sequencing the katanin clones, I concluded that this traditional restriction ligation cloning approach was not successful and thus, I employed an alternative approach. The traditional restriction digestion and ligation cloning was not attempted with spastin.

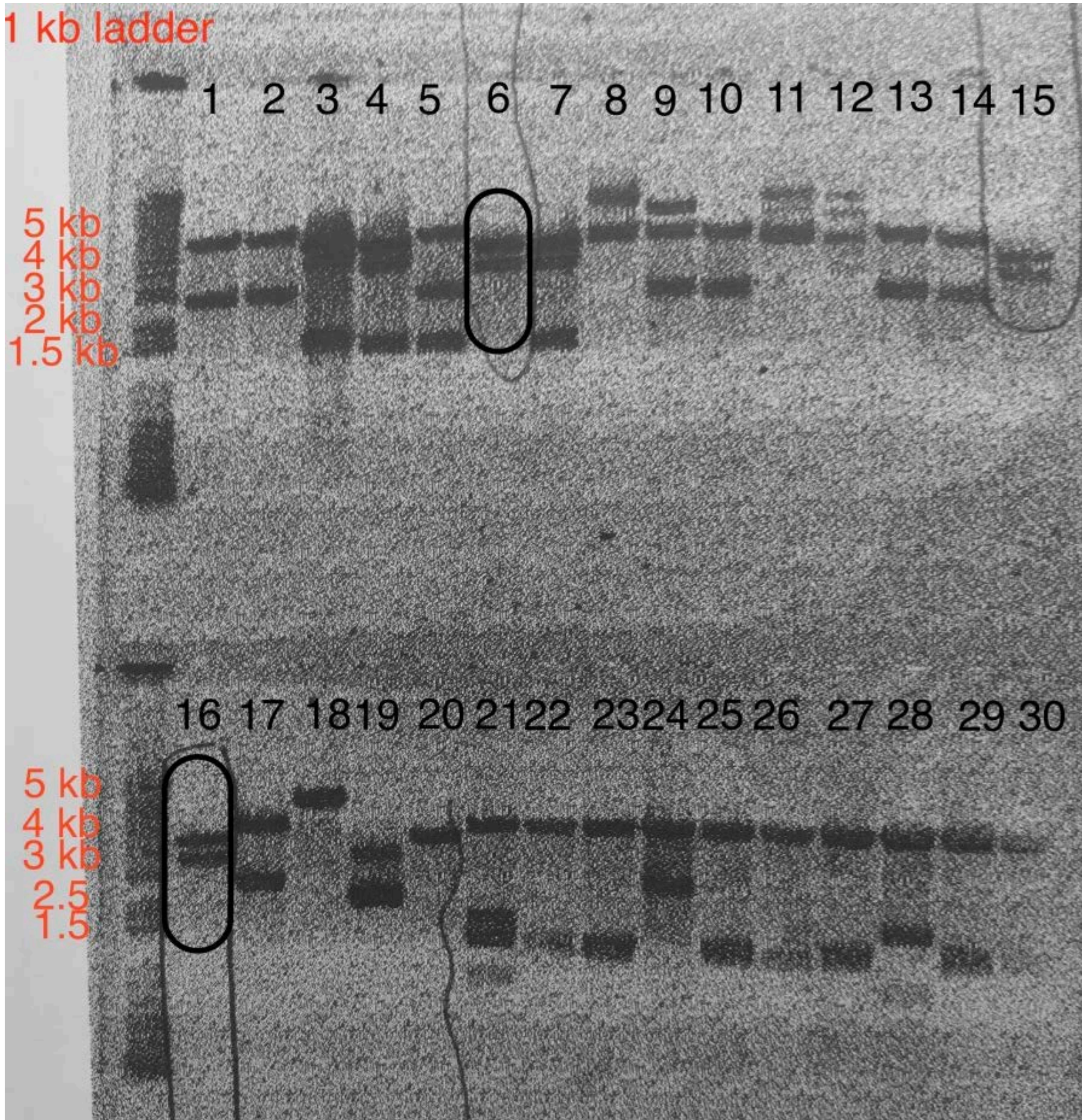

Figure 9. Digestion of pET42a+katanin. 2/30 samples (#6 and #16 circled above) showed the expected fragment sizes of 3.5 kb and 4 kb after digestion with *HpaI*.

The alternative approach was to clone katanin-p60 and spastin to pET42a using In-Fusion cloning. The In-Fusion enzyme recognizes and fuses overlapping 15 bp DNA fragments. In this approach, katanin and spastin cDNAs were amplified with primers that add 15 bp of vector sequence to each end of the amplicon. Next, the PCR product was mixed with linearized vector, in the presence of the In-Fusion enzyme to produce the desired products.

Katanin-p60 and spastin cDNA containing vectors generated by the In-Fusion process were transformed into E. coli cells and subsequent minipreps were done to extract the plasmids. Restriction digests of isolated plasmids showed the presence of the expected sized bands (Fig.10) and those that were sequenced were found to be mutation free. This approach allowed for the successful directional insertion of the katanin and spastin ORFs in frame with the GST ORF in the pET42a vector (Fig. 11 and 12).

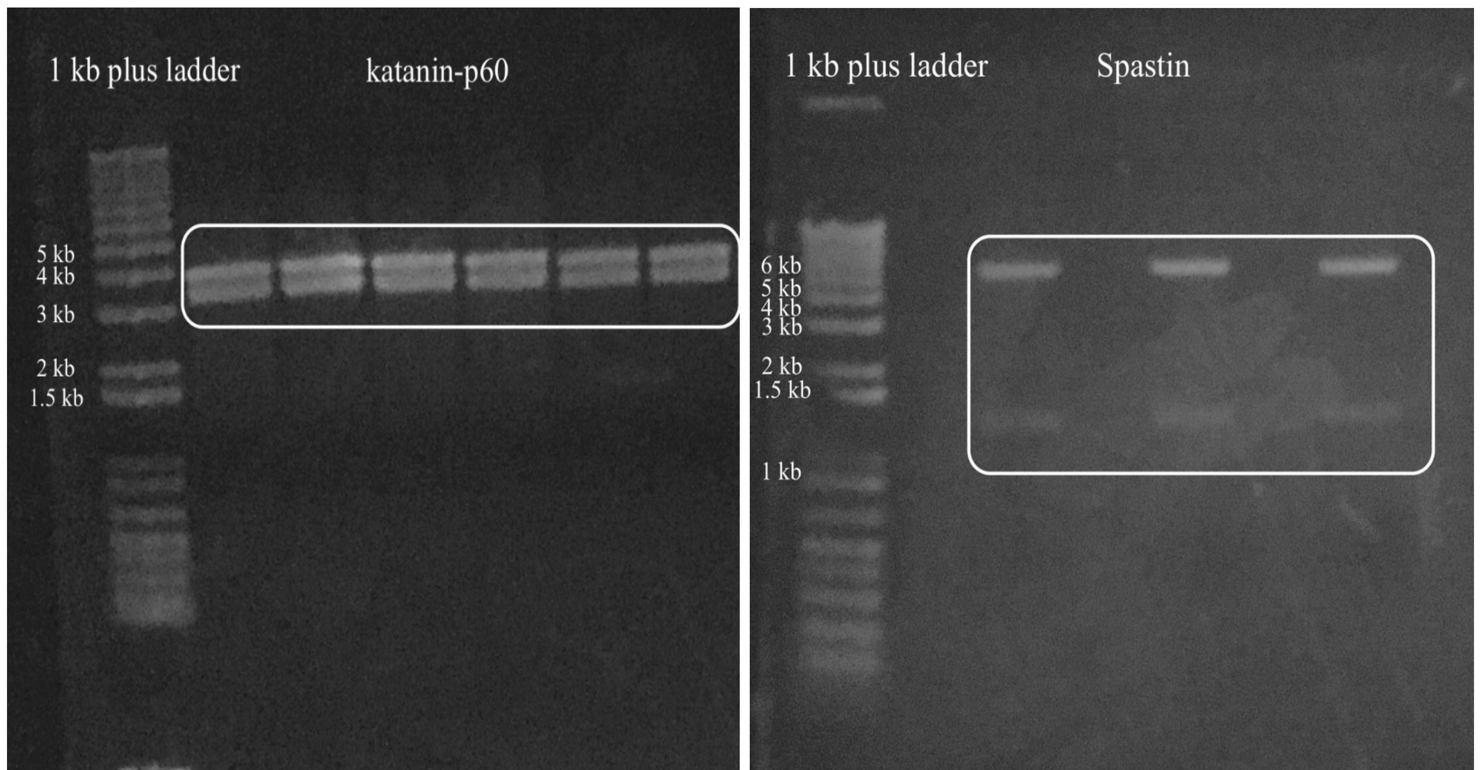

Figure 10. Digestion of the pET42a vector containing katanin-p60 or spastin cDNAs after In-fusion cloning. All transformants analyzed contained the pET42a vector with katanin or spastin inserts, as is evident from the fragments produced by restriction digest with *HpaI* or *NcoI*. The left panel shows the expected 4 kb and 3.5 kb bands after the pET42+katanin constructs were digested with *HpaI*. The right panel shows the expected 6 kb and 1.4 kb bands after the pET42+spastin constructs were digested with *NcoI*.

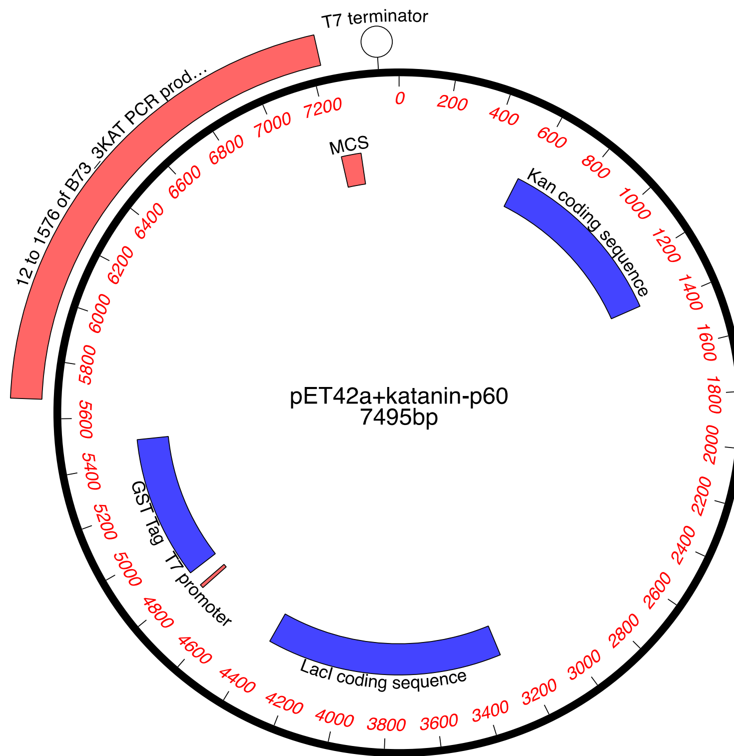

Figure 11. pET42a + katanin-p60 which encodes a GST-katanin protein for expression in *E. coli*.

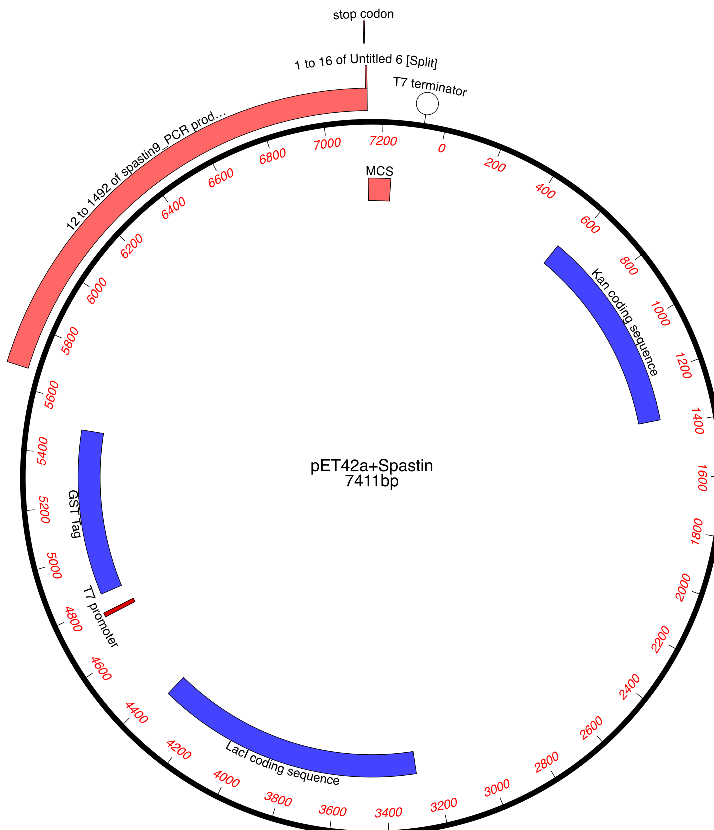

Figure 12. pET42a + spastin vector which encodes a GST-spastin protein for expression in *E. coli*.

Katanin and Spastin Recombinant Protein Expression

In order to express the GST tagged katanin-p60 and spastin proteins, *E. coli* BL21 (DE3) cells were transformed with the pET42a+katanin-p60 and pET42a+spastin vectors (Fig. 11 & 12). *E. coli* was selected as the expression system due to advantages such as fast-growing time, high yields and inexpensiveness. Also, BL21 is one of the most commonly used bacterial hosts and has many advantages such as lacking Lon protease which can degrades foreign proteins (Gottesman et al. 1996). BL21 (DE3) contains a T7 RNA polymerase that recognizes the bacteriophage T7 promoter and allows for high-level expression of recombinant proteins under its control.

After transformation into BL21 (DE3), protein expression was induced by adding IPTG (iso- propyl 1-thio-beta-D-galactopyranoside) to the culture. IPTG induces the expression of T7 RNA polymerase which induces the expression of GST-katanin-p60 and GST-spastin. Different concentrations of IPTG, growing times and growing temperatures were tried to identify the culturing conditions that maximize the production of the protein of interest, but minimize problems like protein degradation and coproduction molecular chaperone.

For katanin-p60, the best culture conditions were 16°C and 0.4 mM IPTG for 48 hours. Following SDS-PAGE of induced cells, a band of the expected size (~ 88 kDa) was seen after Coomassie blue staining of the gel (Fig. 13). To confirm the identity of the 88 kDa band, proteins on a similarly run SDS-PAGE gel were transferred to a membrane via Western blot (Fig. 14). Immunodetection using an anti-GST primary antibody and alkaline phosphatase secondary antibody also highlighted a band of the expected size suggesting that the induced protein is the GST-tagged katanin. Degradation products were also visible on the immunoblot. However, the

use of a protease inhibitor cocktail in subsequent experiments decreased the degradation products significantly (Fig. 16).

For spastin, the best culture conditions were growth at 14°C for 48 hours in the presence of 0.1mM IPTG. Following SDS-PAGE of induced cells, a band of the expected size (~ 85 kDa) was seen on a gel stained with Coomassie blue (Fig. 13) and on a Western blot of a similar gel incubated with an anti-GST primary antibody and alkaline phosphatase secondary antibody (Fig.15). Protease inhibitors were added to spastin as well. These results suggest that I successfully expressed both GST-katanin and GST-spastin in *E. coli*.

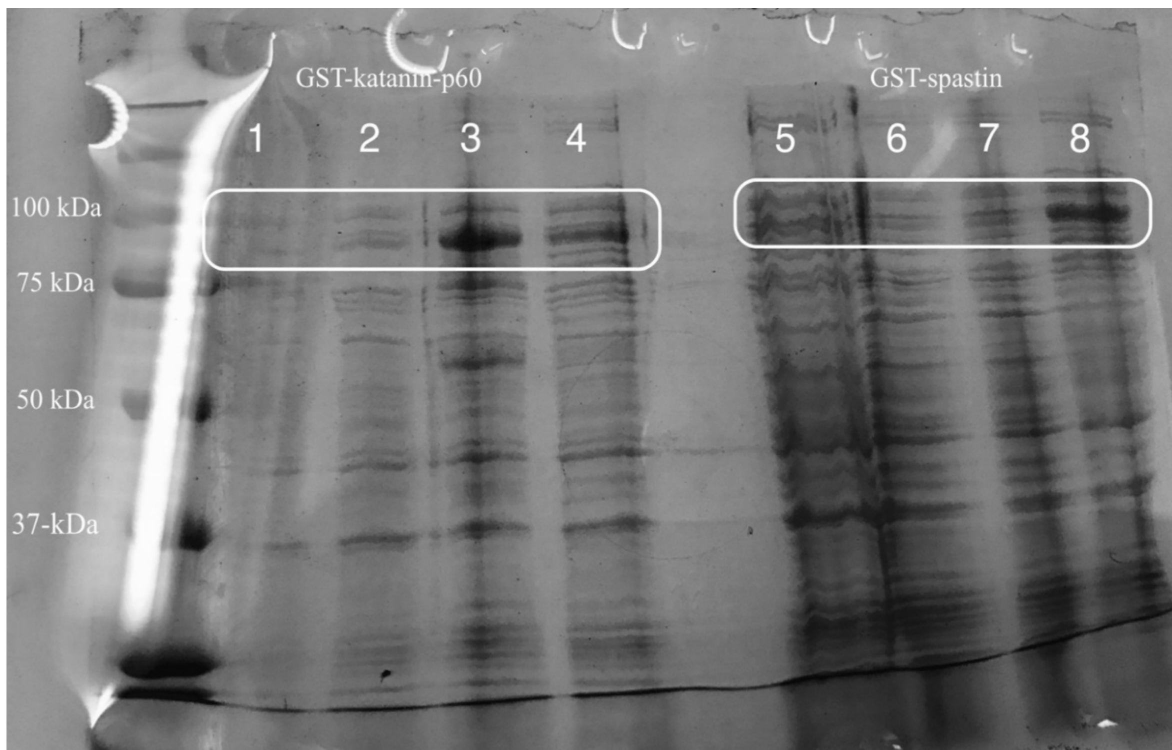

Figure 13. SDS-PAGE gels of GST-katanin and GST-spastin expressing *E. coli* cells. Proteins were visualized by Coommasie blue staining of the gels. Lanes 1-4 are pET42+GST-katanin-p60 (88 kDa) while lanes 5-8 are pET42+GST-spastin (85 kDa). Lane 1: uninduced cultures grown for 48 hours at 16°C. Lane 2: uninduced cultures grown for 72 hours at 16°C. Lane 3: IPTG induced cultures grown for 48 hours at 16°C. Lane 4. IPTG induced cultures grown for 72 hours at 16°C. Lane 5. Uninduced cultures grown for 24 hours at 14°C. Lane 6: uninduced cultures grown for 48 hours at 14°C. Lane 7: IPTG induced cultures grown for 24 hours at 14°C. Lane 8. IPTG induced cultures grown for 48 hours at 14°C. The boxed indicate the GST tagged proteins. Ladder: Molecular weight marker is on the left Precision Plus Protein Standard (BIO-RAD™).

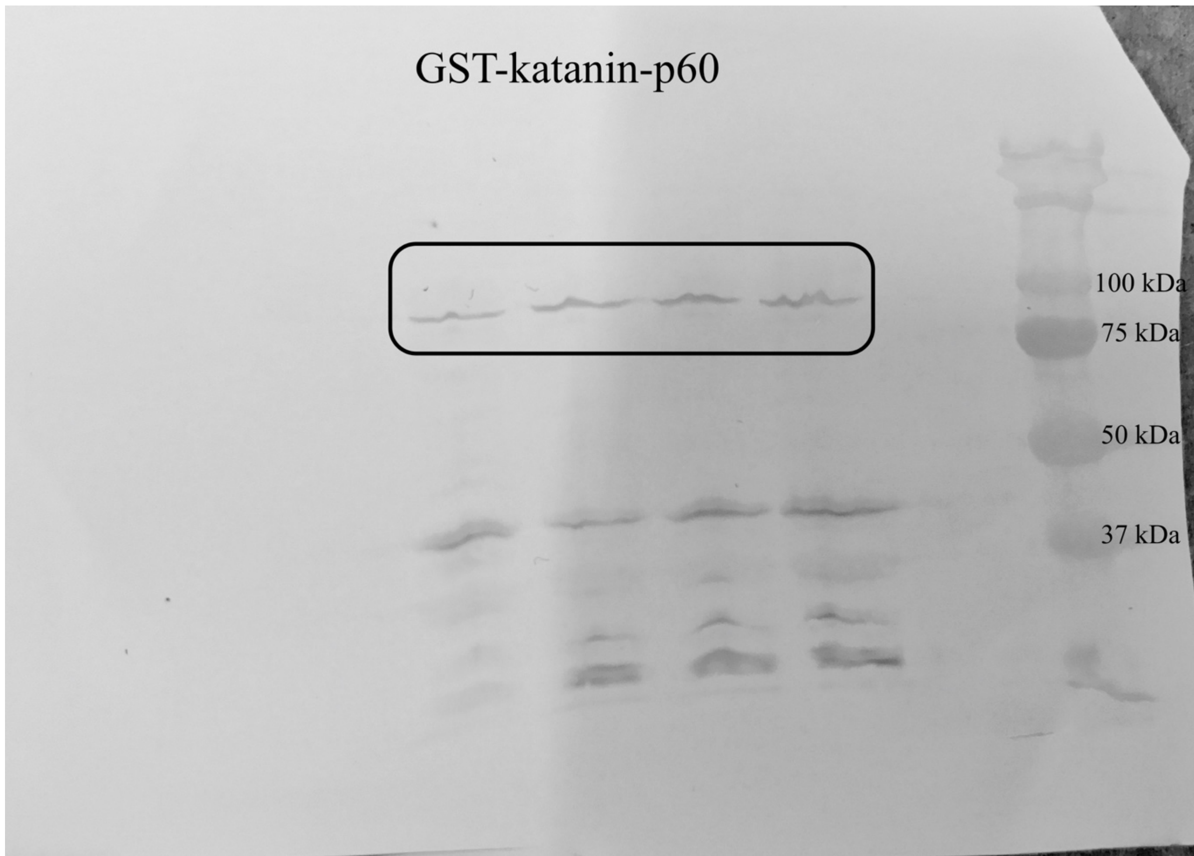

Figure 14. Immunodetection with an anti GST antibody and alkaline phosphatase secondary antibody of induced *E. coli* cells expressing GST-tagged katanin-p60. The boxed region indicates bands the expected size of induced GST-katanin (~88 kDa). Molecular weight marker is on the right. The boxed indicate the same samples Molecular weight marker is on the left Precision Plus Protein Standard (BIO-RAD™).

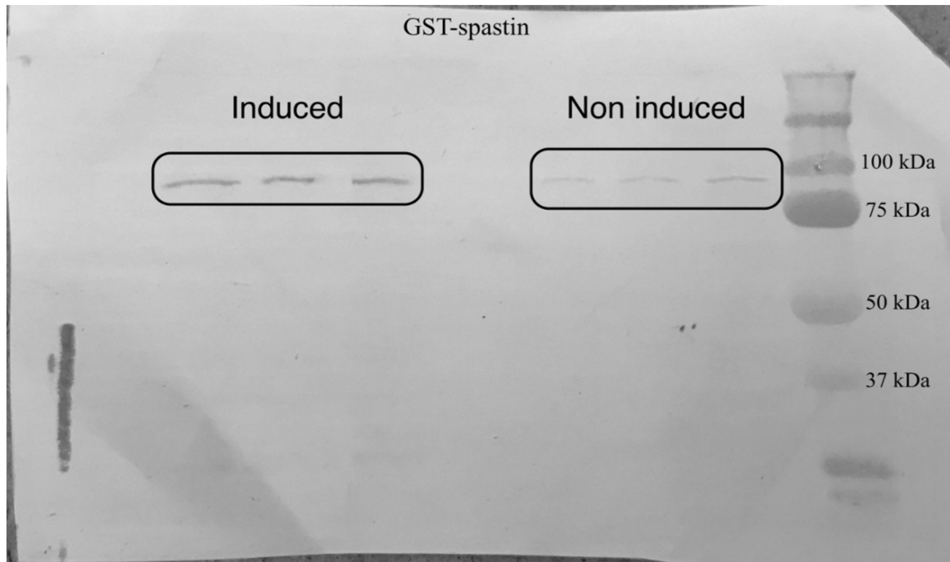

Figure 15. Immunodetection with an anti GST antibody and alkaline phosphatase secondary antibody of induced and uninduced *E. coli* cells expressing GST-tagged spastin. The boxed region indicates bands the expected size of GST-spastin (~85 kDa). Lanes on the left contain proteins from cells induced with IPTG, while lanes on the right contain protein bands from non-induced cells. Precision Plus Protein Standard (BIO-RAD™).

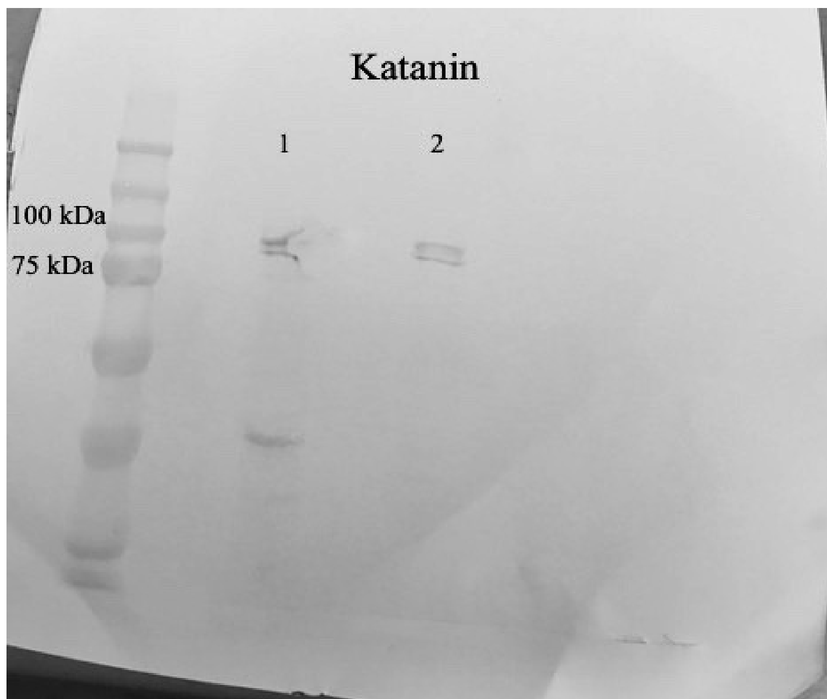

Figure 16. Immunodetection with an anti GST antibody and alkaline phosphatase secondary antibody of induced *E. coli* cells expressing GST-tagged katanin. Lane 1: proteins from induced cells not treated with protease inhibitors. Lane 2: proteins from induced cells treated with protease inhibitors. Molecular weight marker is on the left Precision Plus Protein Standard (BIO-RAD™).

Protein Extraction and Purification

After I determined that I successfully expressed recombinant GST-katanin and GST-spastin into *E. coli,* the next step was to extract and purify the GST-tagged proteins. The *E. coli* cells were lysed by enzymatic digestion and total cell extracts were passed through GSTrap FF columns (Glutathione Sepharose Fast Flow columns) which bind to GST tagged proteins allowing the rest of bacterial cellular proteins to pass through the column. After column washing to remove remaining cellular proteins, the final step was to elute the GST-bound proteins with reduced glutathione. The eluted proteins were run on SDS-PAGE gel and visualized with Coomassie Blue as well as western blotting and immunodetection (Fig. 17 and 18).

For GST-katanin and GST-spastin around 200 ml of cultures were grown, and after proteins expression and purifying the protein, about 2-4 mg/ml proteins concentration was obtained.

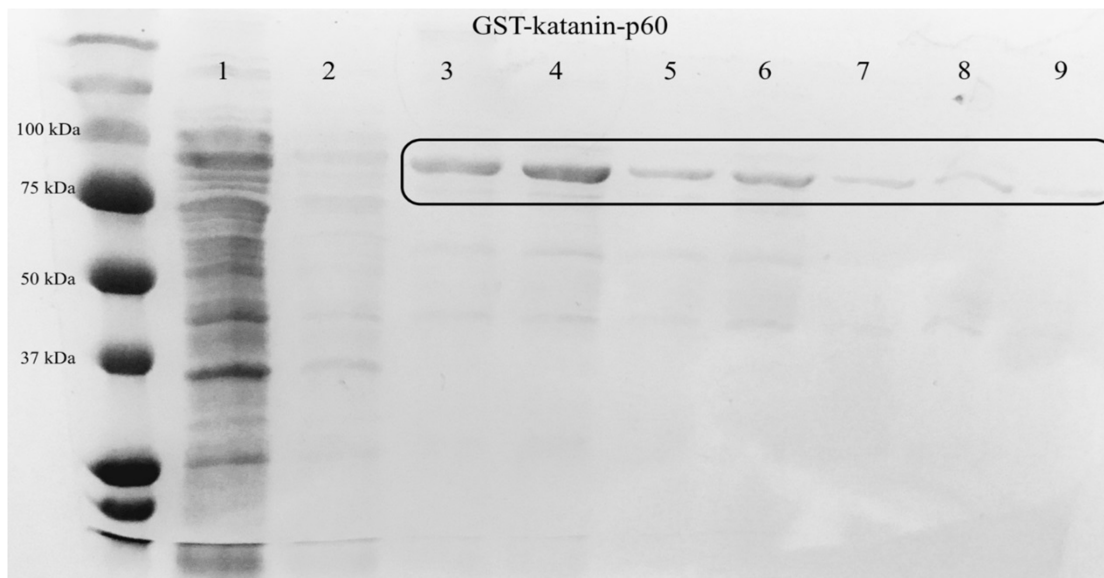

Figure 17. Purified GST- katanin on an SDS-PAGE gel stained with Coomassie Blue. Lane 1: Total cell extracts. Lane 2: Cellular proteins washed from the column. Lanes 3-7: Column fractions of proteins eluted from the column. Boxed region indicates an 88 kDa band the expected size of GST-katanin. Molecular weight marker is on the left Precision Plus Protein Standard (BIO-RAD™).

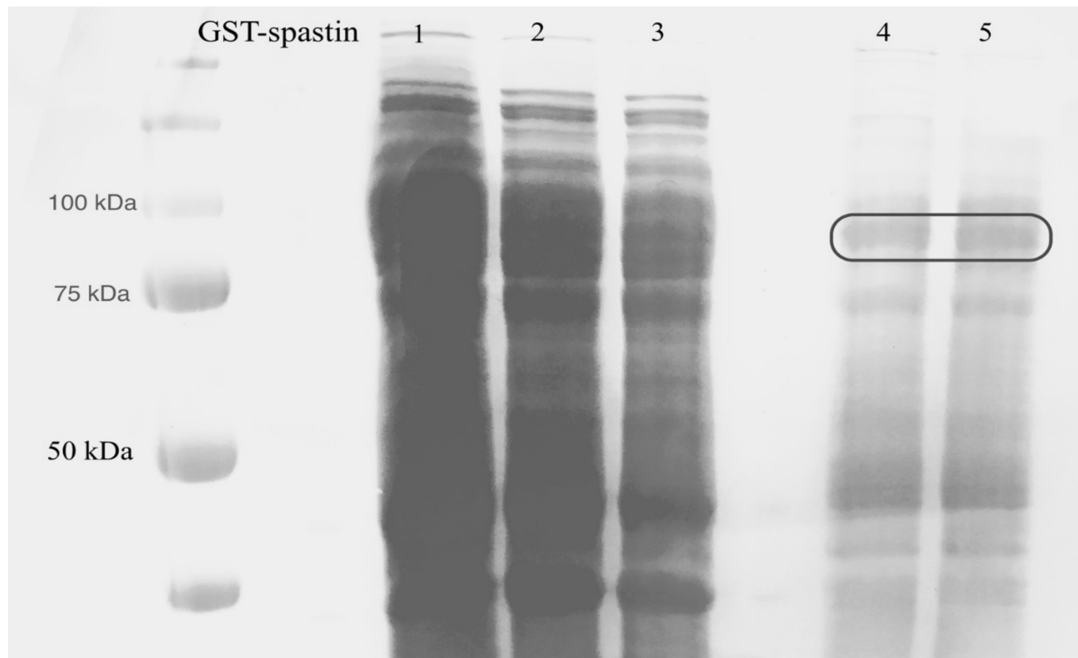

Figure 18. Purified GST- spastin on an SDS-PAGE gel stained with Coomassie Blue. Lane 1: Total cell extracts. Lane 2-3: Cellular proteins washed from the column. Lane 4-5: Purified GST-spastin (85 kDa) Molecular weight marker is on the left Precision Plus Protein Standard (BIO-RAD™).

GST- Cleavage by Factor XA Protease

After successfully expressing and purifying the GST-tagged proteins, I attempted to cleave the GST-tag from the katanin-p60 proteins. The pET42a vector includes a factor Xa protease site between the protein of interest and GST tag. For factor Xa protease cleavage, the optimal cleavage conditions must be determined individually for each protein. However, in a small-scale experiment, cleaving the GST tag appeared to cause the katanin-p60 proteins to become unstable. I tried different incubation lengths, varying concentrations of Xa factor, and a variety of buffers. No conditions resulted in recovery of proteins, suggesting that the proteins lost their solubility without the GST. Cleavage of the GST tag was not attempted with the spastin proteins.

ATPase Activity Assay

Since initial attempts to cleave the GST tag were unsuccessful, the remainder of the assays were performed with GST-katanin-p60 and GST-spastin. Since katanin-p60 and spastin are ATPases that require the hydrolysis of ATP to sever and disassemble stable MTs, the first *in vitro* assay I used to test the functionality of the proteins was an ATPase assay. ATPase assays are performed to test the activity of proteins involved in transport of substrate across the membrane as well as proteins associated with MTs assembly and disassembly (Sharp et al. 2012). In the assay, suspected ATPase proteins are incubated with ATP then a phosphate indicator is added to measure the amount of P_i released after ATP hydrolysis. The indicator reacts with the free P_i to create a green product whose absorbance at 650nm can be measured via a spectrophotometer. In our assay, the spastin and katanin-p60 were mixed with ATP in the presence and absence of MTs. The experiment included a negative (no ATPase protein included) and positive (a kinesin known to hydrolyze ATP) controls. No detectable P_i release was observed in the control reactions that contained only MTs and ATP in the absence of GST-katanin-p60 or GST-spastin or in reactions that contained MTs and GST-katanin-p60 or GST-spastin in the absence of ATP. P_i release was observed in the control reactions that contained a kinesin known to hydrolyze ATP, MT, and ATP. In the case of experimental reactions that contained ATP and GST-katanin-p60 or GST-spastin, I observed P_i release at all tested concentrations of GST-katanin-p60 and GST-spastin in the presence and absence of MTs indicating that GST-katanin-p60 and GST-spastin are capable of hydrolyzing ATP (Fig. 19 and 20). These results suggest that our recombinant proteins are functional and shows for the first time that a plant spastin hydrolyzes ATP.

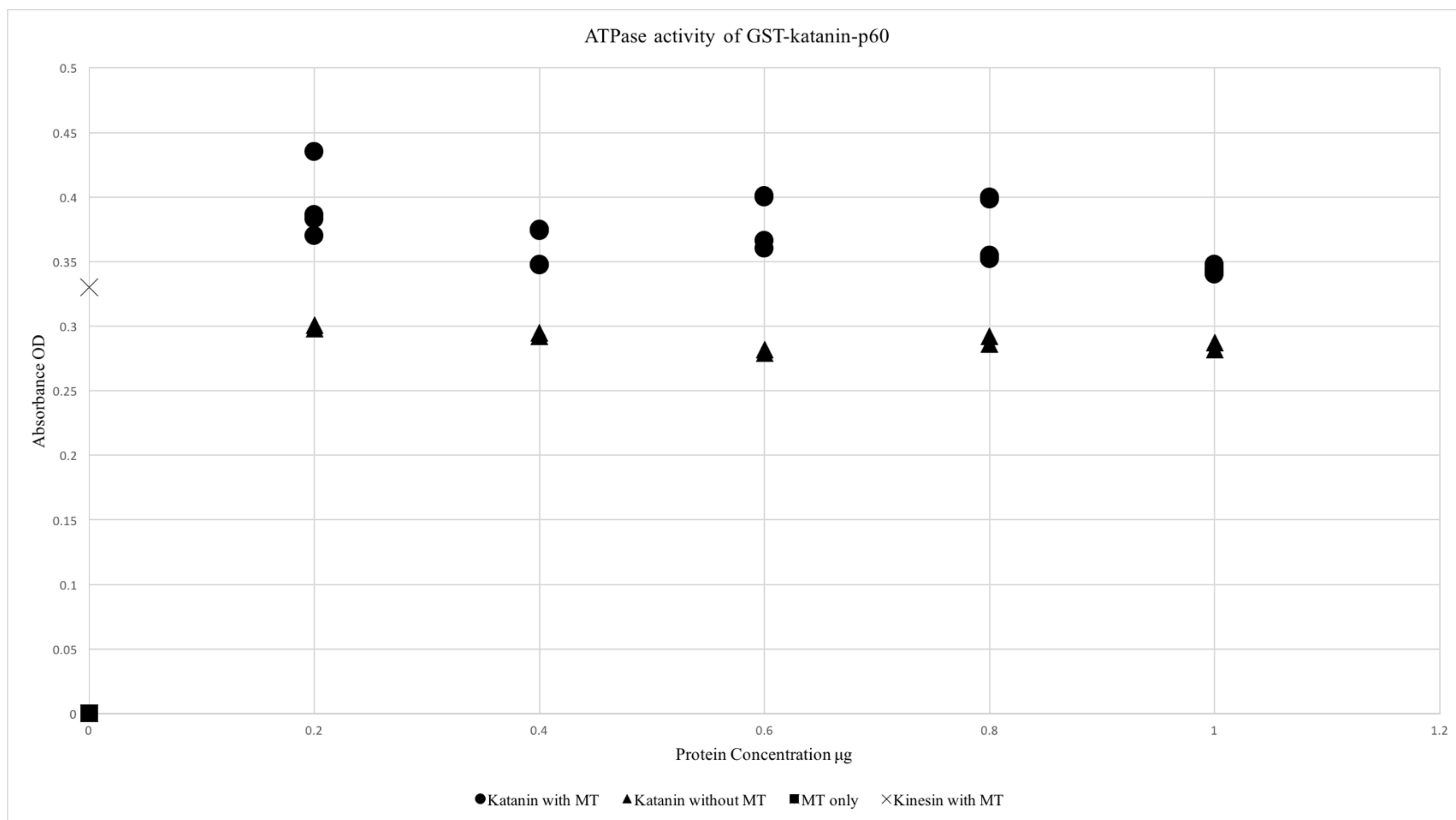

Figure 19. Results of the GST-katanin ATPase activity assay. Circles represent reactions that contained ATP, MTs, and varying concentrations of GST-katanin. Triangles represent reactions that contained ATP and varying concentrations of GST-katanin. Squares represent reactions that contained ATP and MTs only. Xs represent reactions that contain ATP, MTs, and kinesin positive control. Absorbance was measured at 650 nm.

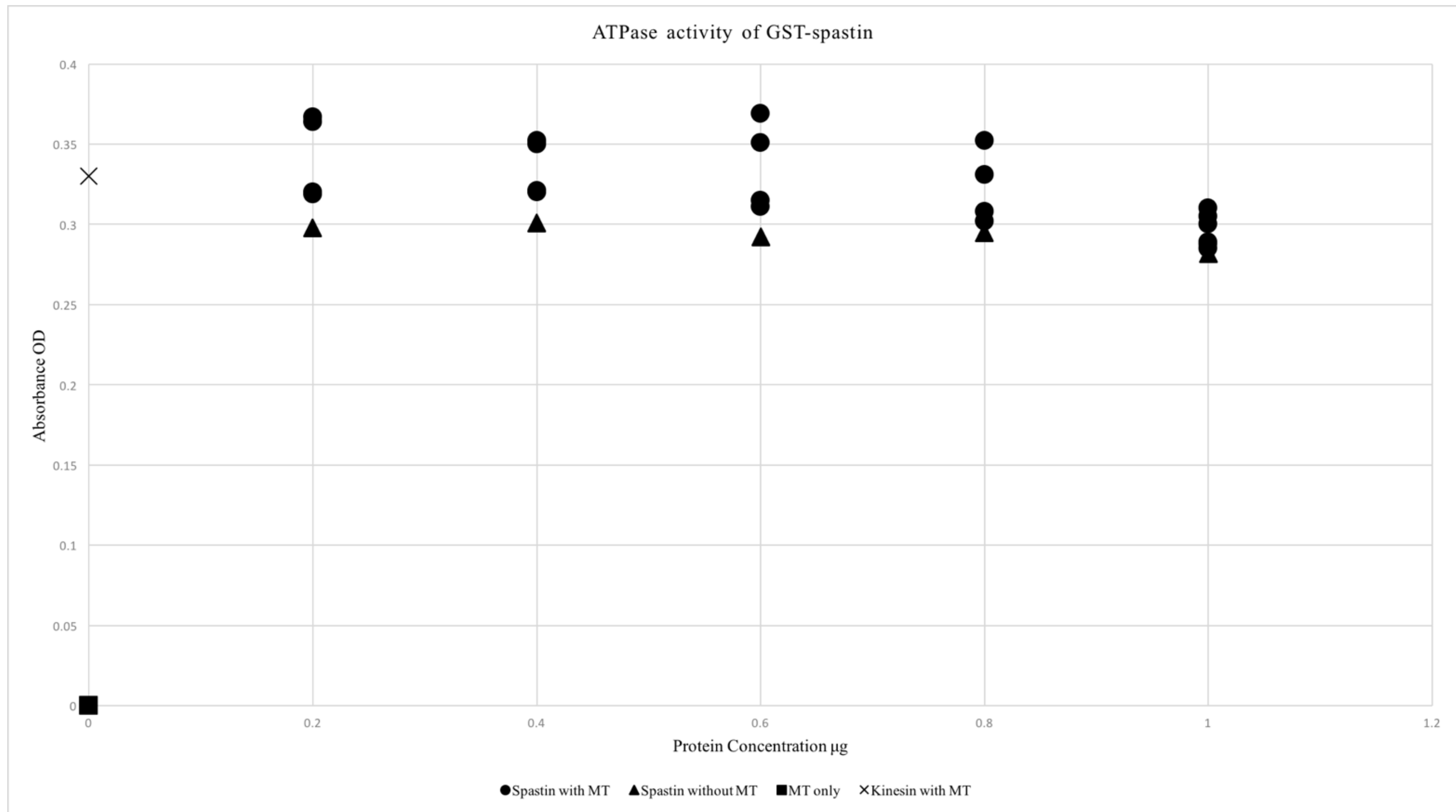

Figure 20. Results of the GST-spastin ATPase activity assay. Circles represent reactions that contained ATP, MTs, and varying concentrations of GST-spastin. Triangles represent reactions that contained ATP and varying concentrations of GST-spastin. Squares represent reactions that contained ATP and MTs only. Xs represent reactions that contain ATP, MTs, and kinesin (positive control). Absorbance was measured at 650 nm.

Tubulin Polymerization Assay

To assess if GST-katanin-p60 and GST-spastin can disrupt MTs, a tubulin polymerization assay was performed. In a tubulin polymerization assay, a tubulin solution is mixed with GTP and spontaneous MT polymerization initiates. As light is scattered by MTs to an extent that is proportional to the concentration of MT polymer, the extent of MT polymerization can be tracked via spectrophotometer over the course of the assay. Thus, if GST-katanin-p60 and GST-spastin are capable of severing MTs, we would expect to see drop in OD value relative to polymerization reactions that do not contain GST-spastin and GST-katanin-p60. The types of reactions that were set-up included control reactions that contained tubulin and GTP or tubulin, GTP, and ATP. Other controls contained tubulin, GTP, and paclitaxel, a drug that promote MT polymerization or tubulin, GTP, and $CaCl_2$ which inhibits MT polymerization. The experimental reactions contained tubulin, GTP, ATP and different concentrations of GST-katanin-p60 and GST-spastin.

The polymerization control (tubulin and GTP) achieved a V_{max} [340] of 20.8. The reaction containing tubulin, GTP and paclitaxel showed the expected elimination of the nucleation phase of MT polymerization, an enhanced V_{max} [340] of 36.1 and an increase in the overall polymer mass of the reaction. Inclusion of $CaCl_2$ with tubulin and GTP reduces the V_{max} [340] of 3.3 and decreases polymer mass. Since ATP is needed for katanin-p60 and spastin function, I included an additional control reaction that contained tubulin, GTP and ATP which achieved a V_{max} [340] of 13.5. Surprisingly, this V_{max} was lower than that observed for the reaction that contained only GTP and tubulin. The experimental reactions contained tubulin, GTP and different concentrations of GST-katanin-p60 and GST-spastin. For GST-spastin, at 0.2 µg, 0.5 µg, and 1 µg the V_{max} [340] was 10.4, 9.1, 8.3 respectively, while for GST-katanin-p60,

at 0.2 μg, 0.5 μg, and 1 μg the V_{max} [340] was 12.9, 11.6, and 9.4 respectively. (Figures 21 and 22).

Our preliminary results indicate a lower V_{max} and a lower total polymer mass in the samples containing GST-katanin-p60 and GST-spastin relative to the control reactions which contained only ATP, GTP and tubulin suggesting that GST-katanin-p60 and GST-spastin may be disrupting MTs via severing. The results need to be sustained by further refinement of the experimental conditions.

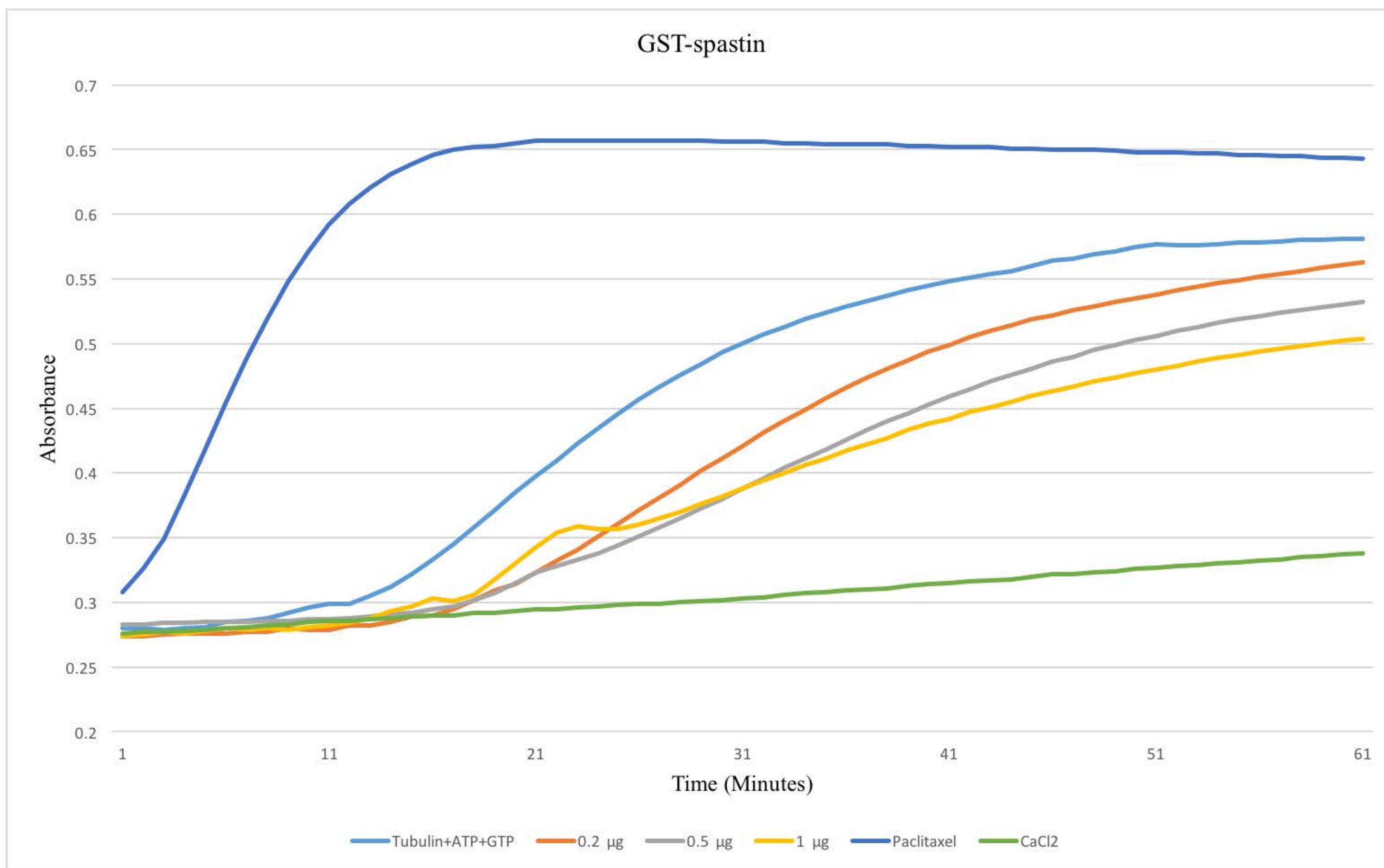

Figure 21. Tubulin polymerization assay of GST-spastin

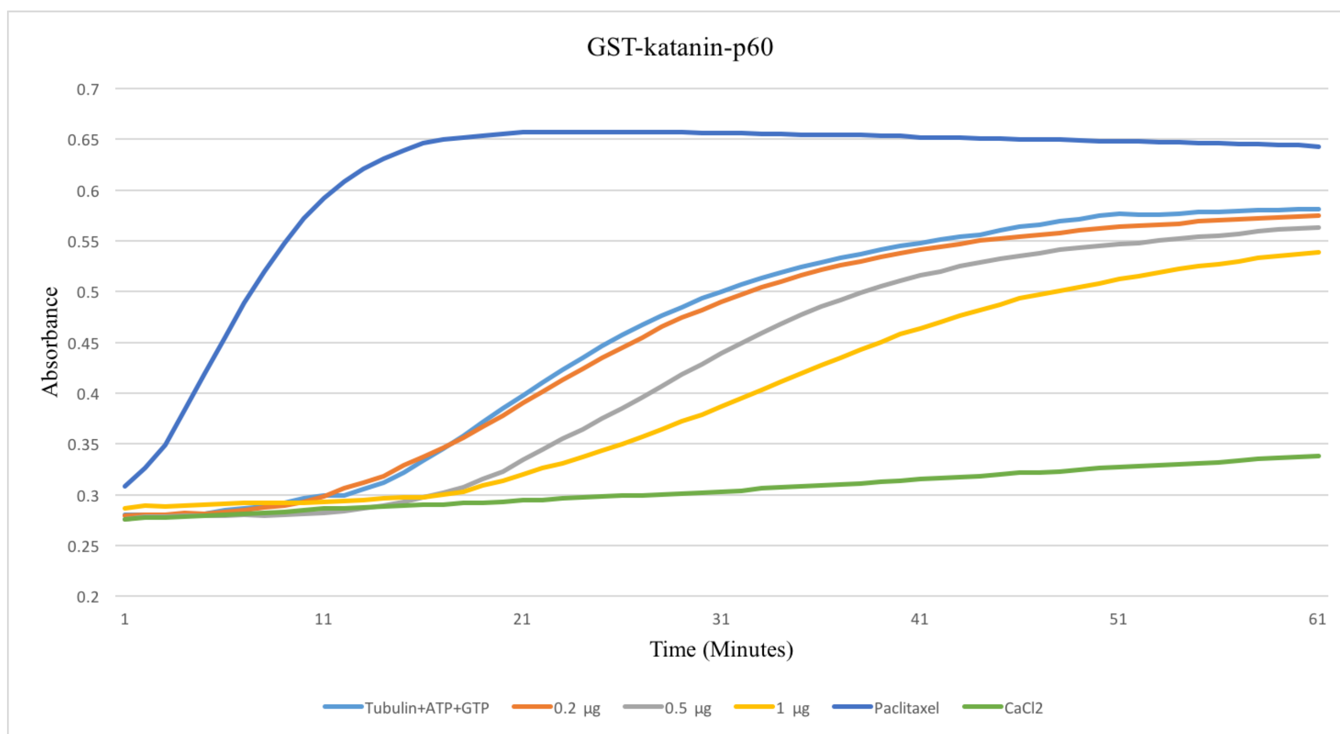

Figure 22. Tubulin polymerization assay of GST-katanin-p60

DISCUSSION

The primary goal of this research project was to generate tools allowing for the expression and purification of the maize spastin and katanin-p60 proteins for *in vitro* analysis. While Arabidopsis katanins are known to sever MTs (Burk et al. 2002. Stoppin-Mellet et al. 2002, Stoppin-Mellet et al. 2006), no information was available about the functionality of maize katanin specifically. Additionally, there is no published reports on any aspect of spastin function in plants at this time.

To achieve the primary goal, maize katanin-p60 (1587 pb) and spastin (1503 bp) cDNAs were cloned into the expression vector, pET42a, in order to generate GST tagged proteins. Initially the traditional restriction enzyme and ligation method was used to generate GST tagged constructs because it is a common cloning method in molecular biology, however, mutation free clones were never identified and thereby this approach was not successful in cloning katanin-p60 and spastin into pET42a vector. Random *E. coli* DNA fragments were inserted instead of the katanin-p60 gene. One possible explanation is that the sticky end of the pET42a vector was prone to interact with *E. coli* genes.

Next, I tried the In-Fusion cloning method which was successfully employed to clone mutation free spastin and katanin-p60 cDNA into the pET42a vector. In-Fusion cloning was rapid, simple, and high efficient in inserting katanin-p60 and spastin-9 genes into the pET42a vector.

GST-katanin-p60 (88 kDa) and GST-spastin (85 kDa) fusion protein expression was successfully induced by culturing transformed *E. coli* in the presence of IPTG. The expression of the fusion proteins were visualized by Coomassie blue staining and immunodetection of the GST tag on a Western blot. However, degradation of these proteins was a challenge for their

expression *in vitro*. The addition of protease inhibitors to the bacterial cell extracts and growing the culture in lower temperature reduced the degradation products. After the vector containing cells were broken open by using the BugBuster reagent, GST-katanin-p60 and GST-spastin were purified by running the bacterial cell extracts over GST-Trap FF columns and eluting the GST-proteins with reduced glutathione.

The results of GST-spastin purification contained many more contaminating bands compared to the results of GST-katanin-p60 purification. To improve purification of GST-spastin, adding another step such as size exclusion chromatography may help. Another approach to improve the purification is to fuse spastin with two different tags, such as a GST and a His tag, on each end of the protein. Doing sequential purifications using first the GST tag and then the His tag could result in final protein population that has a higher percentage of full length GST-spastin.

Initial attempts to cleave the GST tag from the proteins were unsuccessful. The products of the Factor Xa cleavage reactions were separated via SDS-PAGE, but no bands the size of katanin-p60 (60 kDa) was detected on gels stained with either Coomassie blue and silver stain. A band the size of GST was detected however suggesting that GST tag waas cleaved off. One possible explanation for this result is that katanin-p60 and spastin are not soluble under the current purification conditions without the GST tag. Given these results, the protein functionality assays were conducted using GST-katanin-p60 and GST-spastin.

Since katanin-p60 and spastin are both AAA ATPase enzymes and they hydrolyze ATP, (Evans et al. 2005, Salinas et al. 2005, McNally et al. 1993), an ATPase activity assay was used to test the functionality of the purified GST proteins. The ATPase activity assay measured the ability of GST-katanin-p60 and GST-spastin to hydrolyze ATP *in vitro*. In this assay, GST-

katanin-p60 and GST-spastin were incubated with ATP in the presence and the absence of MTs and release of free phosphate from ATP hydrolysis was measured.

Other researchers have shown that MTs stimulate the ATPase activity of katanin-p60 (Stoppin-Mellet et al, 2006; Hartman et al, 1998). Unlike these experiments, the experiment I performed didn't test different concentrations of MTs to determine the maximum ATPase activity of protein of interest + MT. Our results showed that katanin-p60 and spastin hydrolyze ATP and the absence and presence of MTs and the presence of MT may slightly stimulate the ATPase activity. The MT only negative control had no detectable ATPase activity and the kinesin positive control showed result an ATPase activity similar to katanin-p60 and spastin. These results suggest that the ATPase domains of *in vitro* expressed and purified GST-tagged proteins are functional.

Our next experiment to test the functionality of the GST-tagged proteins employed a tubulin polymerization assay. Different concentrations of katanin-p60 and spastin were mixed with ATP, GTP, and a tubulin solution. As the MT polymerization initiates, the extent of this polymerization was tracked via spectrophotometer. The ability of GST-spastin and GST-katanin-p60 to sever MTs was monitored as OD value dropped relative to polymerization reactions that did not contain GST-spastin and GST-katanin-p60. This experiment included a paclitaxel control which enhances tubulin polymerization and $CaCl_2$ which inhibits tubulin polymerization. Surprisingly, comparing the reactions that contain tubulin and GTP to reactions that contain tubulin, GTP, and ATP, showed that the inclusion of ATP slowed the microtubule polymerization rate. One possibility is that ATP is competing for GTP at the active site of tubulin which utilizes GTP hydrolysis as a source of energy for the tubulin polymerization. While the addition of GST-katanin-p60

or GST-spastin to reactions containing tubulin, GTP, and ATP further depressed the V_{max} and the total accumulation of MT polymers, this result needs further confirmation by including additional controls such as adding a non-severing protein to a reaction containing tubulin, GTP, and ATP to examine if adding any protein at the concentrations used alone interfere with the MT polymerization reaction.

In the future, the ability of maize katanin-p60 and maize spastin to sever MTs can also be investigated via a different experimental set up. GST-spastin or GST-katanin are incubated with fluorescent MTs and the MTs are observed via confocal microscopy to look for breaks caused by the action of the severing proteins.

If katanin-p60 and spastin recombinant proteins fail to sever MTs in the fluorescent MT assay, it is possible that the GST tag is interfering with protein activity. Further attempts to cleave the GST tag using different buffers and reaction conditions to stabilize the GST free proteins could be explored. Alternatively, cloning the GST at C terminus could be considered. Another possible reason is that the proteins produced in *E. coli* may not be functional. This issue has been documented with other katanin proteins (Stoppin-Mellet et al. 2002, McNally et al. 2000). An alternative expression system to try is an insect cell system (sf9) which has been shown to produce functional Arabidopsis katanin proteins (Stoppin-Mellet et al. 2002, McNally et al. 2000).

After fully functional MT-severing proteins are obtained, future lines of experimentation include *in vitro* assays to assay the MT severing abilities of various mutant katanin-p60 and spastin alleles. Also, an investigation into if including the katanin-*p80* subunit enhances the activity of katanin-p60 in *in vitro* assays (as seen in Arabidopsis) could be fruitful (McNally et al. 2000).

APPENDIX

EXPERIMENTAL MATERIALS

Bacteria Strains:
Escherichia coli
High Efficiency NEB 5-alpha Competent *E. coli* (New England Biolabs), BL21 (DE3) competent *E.coli* (New England Biolabs™)

Enzymes:
Restriction Enzymes: *EcoRI, EcoRV, BamHI, PshAI, NotI, HindIII, XbaI, , PvuII,* (New England Biolabs™)

Other Enzymes:
Taq DNA polymerase (NEB), Ex Taq DNA polymerase (Takara Bio Inc™).
Vectors: pET-42a (+) DNA (Novagen™), pGEM-T Easy (Promega™),

Chemicals and Kits
Zymoclean Gel DNA recovery kit (Zymo Research™), ZR Plasmid Miniprep- Classic kit, and Zyppy Plasmid Midiprep kit (Zymo Research™), PierceTM Silver Stain Kit (Fisher Scientific™).
BugBuster® 10X Protein Extraction Reagent (Millipore Sigma™). GE Healthcwere GSTrapTM FF Columns and Glutathione (Fisher Scientific™).

Media and Solutions
LB media (per liter)
10 g of NaCl, 10 g of tryptone, 5 g of yeast extract.
Add ddH_2O to a final volume of 1 liter, 15 g of agar (for plates only).
Autoclave 20 minutes. For plates, left them on room temperature for 16 hours (overnight).
Antibiotics and working concentrations
Kanamycin/ 50 µg/ml
Ampicillin/ 50 µg/ml
IPTG/ 200 mM
Xgal/ 50 µg/ml

Buffers and Solutions
10xPBS
80 g NaCl, 2 g KCl, 14.4 g NaHPO4, 2.4 g KH2PO4. Add ddH2O to 1 L. adjust pH to 7.4 by HCl.
4x Laemmli Sample Buffer
Bought from BIO-RAD company.
4x Resolving Buffer
1.5 M Tris-Cl (pH 8.8),0.4% SDS. Add ddH2O to 500 ml, store in 4°C fridge.
5x Running Buffer
15.1 g Tris base, 72 g glycine, 5 g SDS. Add ddH2O to 1 l, store in 4°C fridge.
4x Stacking Buffer
0.5 M Tris-Cl (pH 6.8), 0.4 g SDS. Add ddH2O to 60 ml, store in 4°C fridge.
1x Transfer Buffer
3 g Tris base, 14.5 g glycine, 200 ml methanol. Add ddH2O to 1 L.

10xTBS
30.3 g Tris base, 90 g NaCl. Adjust pH to 7.4. Add ddH2O to 1L. Autoclave for 20 minutes.
Alkaline Phosphate Buffer 10 ml 1M Tris-Cl (pH 9.5), 3.75 ml 4M NaCl, 1ml 0.5M MgCl2. Add ddH2O to 100ml.
Coomassie Blue Buffer
1 g Coomassie Brilliant Blue (Bio-Rad), 500 ml MeOH, 100 ml Glacial acetic acid. Add ddH2O to 1 L.
Elution Buffer (for GSTrap-FF protein purification)
50 mM Tris-HCl, 10 mM reduced glutathione, PH 8.0. One stage vacuum filtration
Washing (Binding) Buffer 1x PBS, PH 7.3. One stage vacuum filtration
Factor Xa Cleavage
Factor Xa Dilution/Storage Buffer (500 mM NaCl, 20 mM Tris-HCl, 2 mM $CaCl_2$, 50% glycerol, pH 8.0)
10X Factor Xa Cleavage/Capture Buffer (1 M NaCl, 500 mM Tris-HCl, 50 mM $CaCl_2$, pH 8.0)

Sequences
Katanin-p60 (*ktn2*) cDNA sequence: 1587 bp
ATGCATGACCCGCGTCAGCGAATCCCCTCGCGGGGCTGCAGGACCACATCAAGCTT
GCGCGGGATTACGCGCTCGAGGGCCTATACGACACCTCCATAATCTTCTTCGACGGC
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CGAAGACCAACAAGAGGTCAATCTAGTGCAAGAAAATCCTCCCAAGATGGAGCCTG
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GCGACTTTGTGGAGGCTCTGGTGAAGGTCCAGAAGAGTGTCTCGCCTGCAGACATA

GAAAAGCACGAGAAGTGGATGGCTGAGTTTGGATCTGCCTGAGACCCGCGTCTGAT
CG

GST-katanin-p60 amino acid sequence (MW 88 kDa)
MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPYYI
DGDVKLTQSMAIIRYIADKHNMLGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFETLK
VDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLYMDPMCLDAFPKL
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GLVPRGSTAIGMKETAAAKFERQHMDSPDLGTGGGSGIEGRASANPLAGLQDHIKLAR
DYALEGLYDTSIIFFDGAIAQINKHLTTLDDALVRTKWMNCKKAISEEVESVKQLDAQL
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VEALVKVQKSVSPADIEKHEKWMAEFGSA*

Spastin cDNA sequence (1503 bp):
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TGATCAGGCCAAGCTTGCAGAAGAGCAAATGGGACGAGCTGGAGAACTGGAACGA
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GST-spastin protein sequence (85 kDa):
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GSS*

Table A.1. List of the primers sequences

Primer name	Primer sequence
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Spas9_Psh_rev	GACGCGGGTCTCAGCTCGAACCGAATTCCT
SpascDNAafor	GCCAAGCATCTACACATCAG
spastin_IF_for	GGTATTGAGGGACGCAGCTTTCTTCGCTCGCTAGC
spastin_IF_rev	ATATCCCATGGACCCGGGTCTCAGCTCGAAC
3kat_Psh_for	GACCCGCGTCAGCGAATCCCCTCGCGGGG
3kat_Psh_rev	GACGCGGGTCTCAGGCAGATCCAAACTCAG
3KATcDNAhfor	GGAGGCTCTGTGGTTAAATC
IF_3KATfor	GGTATTGAGGGACGCGCGTCAGCGAATCCCCTCGCGGG
IF_3KATrev	ATATCCCATGGACCCGGGTCTCAGGCAGATCCAAAC

REFERENCES

Burk, D. H., & Ye, Z. (2002). Alteration of Oriented Deposition of Cellulose Microfibrils by Mutation of a Katanin-Like Microtubule-Severing Protein. *The Plant Cell Online,* 14(**9**), 2145-2160.

Burk, D. H., Liu, B., Zhong, R., Morrison, W. H. and Ye, Z. H. (2001). A katanin-like protein regulates normal cell wall biosynthesis and cell elongation. *Plant Cell* **13**, 807-827.

Evans K. J., Gomes E. R., Reisenweber S. M., Gundersen G. G., Lauring B.P. (2005). Linking axonal degeneration to microtubule remodeling by spastin-mediated microtubule severing. *Journal Cell Biol*, **168**, 599-606.

Frickey, T., Lupas, A. N. (2004). Phylogenetic analysis of AAA proteins. *J Struct Biol*, **146**, 2-10.

Gottesman, S. (1996). Proteases and Their Targets In *Escherichia Coli*. *Annual Review of Genetics*, 30(**1**), 465-506.

Hartman J. J., Mahr J., McNally K., Okawa K., Iwamatsu A., Thomas S., Cheesman S., Heuser J., Vale R. D., McNally F. J. (1998). Katanin, a microtubule-severing protein, is a novel AAA ATPase that targets to the centrosome using a WD40-containing subunit. *Cell*, (**93**), 277-287.

Hazan, J., N. Fonknechten, D. Mavel, C. Paternotte, D. Samson, F. Artiguenave, C.S. Davoine, C. Cruaud, A. Durr, P. Wincker (1999). Spastin, a new AAA protein, is altered in the most frequent form of autosomal dominant spastic paraplegia. *Nature Genetics.* (**23**), 296–303.

Komorisono, M., Ueguchi-Tanaka, M., Aichi, I., Hasegawa, Y., Ashikari, M., Kitano, H., Sazuka, T., (2005). Analysis of the Rice Mutant dwarf and gladius leaf 1. Aberrant Katanin-Mediated Microtubule Organization Causes Up-Regulation of Gibberellin Biosynthetic Genes Independently of Gibberellin Signaling. *Plant Physiology*, 138(**4**), 1982-1993.

McNally, K. P., Bazirgan, O. A., & McNally, F. J. (2000). Two domains of p80 katanin regulate microtubule severing and spindle pole targeting by p60 katanin. *Journal of Cell Science*, 113(**1623**)

Mains, P. E., Kemphues K. J., Sprunger S. A., Sulston I. A., Wood, W. B. (1990). Mutations affecting the meiotic and mitotic divisions of the early *Caenorhabditis elegans* embryo. *Genetics*, **126**, 593-605.

McNally F. J., Vale R. D. (1993). Identification of katanin, an ATPase that severs and disassembles stable microtubules. *Cell*, **75**, 419-429.

Murata, T., Sonobe, S., Baskin, T. I., Hyodo, S., Hasezawa, S., Nagata, T., Horio, T., Hasebe, M. (2005). Microtubule-dependent microtubule nucleation based on recruitment of gamma-tubulin in higher plants. *Nature Cell Biology*, **7**, 961-968.

Nakamura, M., Hashimoto, T. (2009). A mutation in the Arabidopsis {gamma}-tubulin-containing complex causes helical growth and abnormal microtubule branching. *Journal of Cell Science,* **122**, 2208-2217.

Qu, J., Ye, J., Geng, Y., Sun, Y., Gao, S., Zhang, B., Chua, N. (2012). Dissecting Functions of KATANIN and WRINKLED1 in Cotton Fiber Development by Virus-Induced Gene Silencing. *Plant Physiology*, 160(**2**), 738-748.

Salinas, S., Carazo-Salas, R. E., Proukakis, C., Cooper, J., Weston, A., Schiavo, G., Warner, T. T. (2005). Human spastin has multiple microtubule- related functions. *Journal of Neurochem*, **95**, 1411-1420.

Sharp, D. J., & Ross, J. L. (2012). Microtubule-severing enzymes at the cutting edge. *Journal of Cell Science*, *125*(**11**), 2561–2569.

Shaw, S. L., Kamyar, R., Ehrhardt, D. W. (2003). Sustained microtubule treadmilling in Arabidopsis cortical arrays. *Science*, **300**, 1715-1718.

Sherwood, N., Sun, Q., Xue, M., Zhang, B., Zinn, K. (2004). Drosophila spastin regulates synaptic microtubule networks and is required for normal motor function. *PLoS Biol,* **2**, 429.

Stoppin-Mellet, V., Gaillard, J., & Vantard, M. (2002). Functional evidence for in vitro microtubule severing by the plant katanin homologue. *Biochemical Journal*, 365(**2**), 337-342.

Stoppin-Mellet, V., Gaillard, J., & Vantard, M. (2006). Katanin's severing activity favors bundling of cortical microtubules in plants. *Plant Journal,* 46(**6**), 1009-1017.

Stoppin-Mellet, V., Gaillard, J., Timmers, T., Neumann, E., Conway, J., & Vantard, M. (2007). Arabidopsis katanin binds microtubules using a multimeric microtubule-binding domain. *Plant Physiology and Biochemistry*, 45(**12**), 867-877.

Trotta, N., Orso, G., Rossetto, M. G., Daga, A., Broadie, K. (2004). The hereditary spastic paraplegia gene, spastin, regulates microtubule stability to modulate synaptic structure and function. *Current Biology*, **14**, 1135-1147.

Wood, J. D., Landers, J. A., Bingley, M., McDermott, C. J., Thomas- McArthur, V., Gleadall, L. J., Shaw, P. J., Cunliffe, V. T. (2006). The microtubule-severing protein spastin is essential for axon outgrowth in the zebrafish embryo. *Human Molecular Genetics*, **15**, 2763-2771.

Yu, W., Qiang, L., Solowska, J. M., Karabay, A., Korulu, S., Baas, P. W. (2008). The microtubule-severing proteins spastin and katanin participate differently in the formation

of axonal branches. *Mol Biol Cell*, **19**, 1485-1498.

Zhang, D., Rogers, G. C., Buster, D. W., Sharp, D. J. (2007). Three microtubule severing enzymes contribute to the ''Pacman-flux'' machinery that moves chromosomes. *Journal of Cell Biol,* **177**, 231-242.