Title

The utilization of genetic modification and biochemical analysis involved in stress response in the model organism Arabidopsis thaliana.

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The utilization of genetic modification and biochemical analysis involved in the stomatal response in the model organism *Arabidopsis thaliana*.

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in

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by

Thien Trac

Committee in charge:

Professor Julian I Schroder, Chair
Professor Alisa Huffaker
Professor Yunde Zhao

2021

The Master's Thesis of Thien Trac is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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Chapter 1 and chapter 2 are co-authored with Dr. Yohei Takahashi. The thesis author was the primary author of these chapters.

ABSTRACT OF THE THESIS

The utilization of genetic modification and biochemical analysis involved in the stomatal response in the model organism *Arabidopsis thaliana*.

by

Thien Trac

Master of Science in Biology

University of California San Diego, 2021

Professor Julian Schroeder, Chair

Abiotic stresses such as drought, salinity, and temperature trigger an increased production of the phytohormone abscisic acid (ABA), which plays several important roles in plant development, such as drought resistance, seed germination, stomatal response, and water use efficiency. A recent study showed that Raf-like MAPKK-kinases (*M3Ks*) are required to activate OST1/SnRK2 protein kinases after PP2Cs-dependent dephosphorylation in the presence of ABA.

However, the function of *M3Ks* in stomata remains largely unknown. Previously no guard cell-targeted gene induction system has been reported. Here, to further examine the role of the *M3Ks* genes, a strategy for overexpression and induction lines was developed and transgenic lines were generated using a strong Guard Cell-specific Promoter (*pGC1*) and green fluorescent protein (*GFP*) tag. The overexpression constructs were created using the Multisite Gateway Cloning technology and inducible versions of *M3Ks* genes have been generated using USER Cloning. Both constructs were transformed into *Arabidopsis thaliana* using *Agrobacterium* transformation. Seeds were collected and transgenic plants went through Basta (overexpression) or Hygromycin (inducible) antibiotic selection, genotyping using DNA extraction, and confocal microscopic analyses to determine green fluorescent protein (*GFP*) activity. Two truncated constitutively active (CA) versions of Raf-Kδ1 constructs were generated using Gateway (overexpression) and USER (ethanol inducible) cloning technology. An increase in ABA concentration has been shown to lead to stomatal closure, the loss of the ability to transpire water will result in the release of water vapor, which will show warmer leaf temperatures and can be measured by thermal imaging. Thermal imaging and stomatal conductance experiments suggest that both overexpression line and inducible line have a higher leaf temperature when compared with the wild type plants and similar temperature of the closed stomata mutant controls “High Leaf Temperature 1” (*ht1-2*). These preliminary data suggest that upon increasing the expression of the M3Ks gene, transgenic plants are able to close stomata more than the wild type, which potentially allows plants to display higher water use efficiency.

Chapter 1 The functionality of Raf-like kinase M3Ks in plant ABA Signaling Pathway in the model organism *Arabidopsis thaliana*.

Introduction:

Unlike humans and animals, plants cannot relocate from one place to another when their environment changes; therefore, they need to adapt to the environments to maintain homeostasis (Raza et al., 2019). For plants to tolerate a stressful environment such as drought, multiple cellular responses need to be activated including the production of the hormone abscisic acid (ABA). ABA is the plant hormone that is crucial for seed germination, development, stress, and drought responses (Ainsworth et al., 2007; Engineer et al., 2016; Kang et al., 2010; Kuromori et al., 2010). Upon receiving the drought signal from the root, ABA hormone is produced and sent to leaf tissue. To understand how plants can respond to current global climate change, studying the roles and mechanism of each component of the ABA phytohormone's pathway is critical.

Plants are known to control gas exchange to increase photosynthesis and minimize water loss to increase water use efficiency (Dettinger et al., 2015). To do so, plants are required to adapt to the diverse and complex environment of biotic and abiotic stress signals. On the surface of the leaves, the stomatal are surround by pairs of guard cells to regulate CO_2, O_2, and water for photosynthesis (Willmer et al., 1996). Based on the environment, guard cells can be stimulated to open and close the stomata (Davies & Zhang et al., 1991). Stomatal closure can be induced by several factors such as the presence of plant hormone abscisic acid (ABA), darkness, increase in CO_2 concentration, decrease in humidity, and the release of ions (Hetherington et al., 2003). Upon closing of stomata, the guard cells are shrinking by opening anion channels to depolarize the plasma membrane, activating the cation channels, and efflux of K^+ ions out of the membrane.

By releasing of cation, the water follows the solute out of the membrane which causes the cells to shrink, as a result, a decrease in turgor pressure of the cells (Daszkowska-Golec & Szarejko, 2013; Kollist et al., 2014; Malcheska et al., 2017). On the other hand, the stomatal opening is triggered by red and blue light, decrease in CO_2 concentration, increase in intake of ion and humidity, which cause the increase in turgidity (Willmer et al., 1996).

In plants, there are two types of stomatal structures for monocots and dicots. However, they have the same function as to facilitate the gas exchange and transpiration of absorption of water from the soil through the xylem. The main difference between the stomata of monocot and dicot plants is that the guard cells of the dicots stomata are kidney (bean) shaped, whereas in monocots (grasses) stomata are dumbbell-shaped and surrounded by subsidiary cells. Up to now, *Arabidopsis thaliana* is the most common organism that is used in plant biology studies since they are small, have a fast life cycle, and extensive genomic resources have been developed (Kaul et al., 2000). The organism contains over 27,000 protein-coding genes encoding around 35,000 proteins (Cheng et al., 2017).

Forward genetic screening approaches have been used in *A. thaliana* to study how abiotic stress activates ABA signaling. The early events in the ABA signaling pathway contain many components such as SNF1-related protein kinase 2s (SnRK2s), Type 2C protein phosphatase (PP2C), ABA Receptors PYR-ABACTIC RESISTANCE (PYR/PYL)/ Regulatory Component of ABA Receptor (RCAR), and MAP kinase kinase kinases (M3Ks) (Ma, Y et al., 2009; Park, S.T. et al., 2009; Takahashi et al., 2020; Tischer, S. V. et al., 2009; Umezawa, T. et al., 2009; Vlad, F. et al., 2010; Weiner, J. J. et al., 2010). In absence of ABA, PYR/PYL/RCAR remaining inactive, PP2C the negative regulators will bind to OST1/SnRK2 which inhibits substrate phosphorylation activity for OST1/SnRK2, resulting in no ABA response.

Upon the presence of ABA, the hormone will bind to Pyrabactin resistance/Regulatory Component of ABA Receptor (PYR/PYL/RCAR) which becomes active and recruits downstream negative regulators known as PP2Cs in the ABA signaling pathway (Hubbard et al., 2010; Park et al., 2009). This inhibits the activity of PP2Cs, leading to the activation of OST1/SnRK2 protein kinase, and triggering a series of events to activate the downstream activity of ABA response resistance genes (Hauser et al., 2017; Ma et al., 2009; Vlad, F. et al., 2010). OST1/SnRK2 protein kinase is plant-specific enzymes that participate in ABA signal transduction and environmental stress signaling (Fujii et al., 2007). OST1/SnRK2 regulates the downstream activity of ABA signaling pathways; but also play roles in ion transport, gene expression, and mediating the regulation of stomatal aperture and metabolism of plants (Bucholc et al., 2010; Hrabak et al., 2003; Mustilli et al., 2002; Yoshida et al., 2002). It was well known that the phosphorylation of the SnRK2 protein kinase is required for stomatal closure in response to the presence of ABA; however, this protein kinase cannot activate itself by auto-phosphorylation after dephosphorylation, which means another protein need to present to activate the OST1/SnRK2 (Takahashi et al., 2020).

In addition to SnRK2, a newly discovered protein involved in the ABA signal transduction pathway- MAP kinase kinase kinases (M3Ks) (Takahashi et al., 2020). In *A. thaliana*, M3Ks is one of the largest groups of the MAPK cascade components, with 80 individuals (MAPK, Trends Plant Science 7, 301-308, 2002). Work by Takahashi et al., 2020 has demonstrated that MAPKKKs are involved in the ABA regulation pathway in Arabidopsis thaliana. From recent studies in the presence of ABA, after PYR/PYL/RCAR receptors bind and inhibit PP2C activity which dephosphorylates OST1/SnRK2; OST1/SnRK2 will then be phosphorylated and activated by the M3Ks protein in response to stress (Takahashi et al., 2020).

Reconstitution of the ABA signaling pathway could not be observed when MAPKKKs were removed. The mutant plants using the CRISPR Cas9 system showed a significantly impaired ABA sensitivity phenotype. In-gel kinase assays showed the three M3Ks (δ1, δ6, δ7) to have strong activation of OST1/SnRK2 after dephosphorylation (Takahashi et al., 2020). While M3Ks genes were shown to participate in the regulation of plant development and the ABA signaling pathway, there is still limited knowledge on the mechanisms and activities of the gene.

In this study, genetic modification, cell imaging, and biochemical analysis were used to analyze transgenic *Arabidopsis* lines to analyze the function of *M3Ks* genes. A hypothesis was investigated in this study that upon increasing the expression in M3K proteins, the phosphorylation of OST1/SnRK2 could be increase; thereby promoting stronger ABA signaling. In order to investigate this hypothesis, transgenic plants will need to be generated using strong guard cell promoters to compare the expression levels and ABA responses with wild-type plants. Thermal imaging is a helpful technique to detect the difference between canopy leaf temperatures of transgenic plants with wild-type control plants. Plants perceive osmotic stress leading to stomatal closure by a decrease in stomatal apertures, which causes an increase in leaf temperature since leaf evapotranspiration would be limited (Osakabe et al., 2013; Zhang et al., 2018). Using infrared thermography allows screening stomatal impairment mutants in a non-invasive manner. If the mutants are identified to have cooler leaf temperatures under known stressful conditions, the mutants might contain causative mutations that serve important roles in stomatal response (Wang et al., 2004). Additionally, intact leaf gas exchange analyses were performed to characterization the transgenic genotype in comparison of stomatal conductance with wild-type and *ht1-2* mutant plants.

Results:

1.1 Raf-like MAPKK-kinases (M3Ks) are required to reactivate OST1/SnRK2 protein kinases after dephosphorylation.

OST1 protein kinase is one of the SnRK2 proteins present in *A. thaliana* which serves as a key component in activating the ABA response (Umezama, T. et al., 2009). To examine whether the OST1/SnRK2 protein kinase able to activate itself after being dephosphorylation in the presence of ABA, in-gel kinase assays were performed and reported by Dr. Yohei Takahashi. The GST- protein-tag was added to dephosphorylate the protein kinase, the recombinant protein of OST1/SnRK2.6 shows expression at ~75 kDa (Figure 1a, lane 1). When comparing the autophosphorylation between OST1/SnRK2.6 versus dephosphorylation of OST1/SnRK2.6 by protein phosphatase, the dephosphorylation complex-GST-OST1/SnRK2.6 shows very weak autophosphorylation activity (Figure 1a, lane 2). Furthermore, to test whether other protein kinases in the ABA signaling pathway were able to phosphorylate OST1/SnRK2.6, calcium-dependent protein kinases CPK6 and CPK23 and MAP kinase MAP12 were added. These protein kinases did not show the dephosphorylate OST1/SnRK2.6 (Takahashi et al., 2020). The two dark bands in Figure 2a, 3rd and 4th lane show the autophosphorylation of CPK6 and CPK23 (Brandt et al., 2015; Geiger et al., 2010; Jammes et al., 2009). This result indicates that another protein kinase is required to reactivate OST1/SnRK2 protein kinase activity after de-phosphorylation when ABA is present.

Since autophosphorylation proved not to be valid for OST1/SnRK2.6, further investigation is required to understand how OST1/SnRK2.6 can be activated after dephosphorylation. In-gel kinase assay was performed on the dephosphorylated His-OST1/ SnRK2.6 with seven M3Ks (At2g42640, At1g73660, At4g24480, At1g08720, At1g11850,

At1g16270, At2g42640 and At1g18160) to compare the OST1/SnRK2.6 activation activity (Takahashi et al., 2020). Interestingly, three of the B3 subgroup family showed strong activation with OST1/SnRK2.6 which are At511850 (m3kδ1), At1g73660 (m3kδ6), and At1g18160 (m3kδ7) (Figure 1b). Further investigation was applied to understand whether the full length or truncated constitutively active of the gene shows any distinction in protein activities from one another. The phosphorylation reactions of GST-OST1/SnRK2.6 were started by adding [γ-32P]-ATP. After 30 minutes of incubation, experiments indicated that the truncated kinase domain version of m3kδ1 showed higher activity compared to the full-length version (Figure 1c).

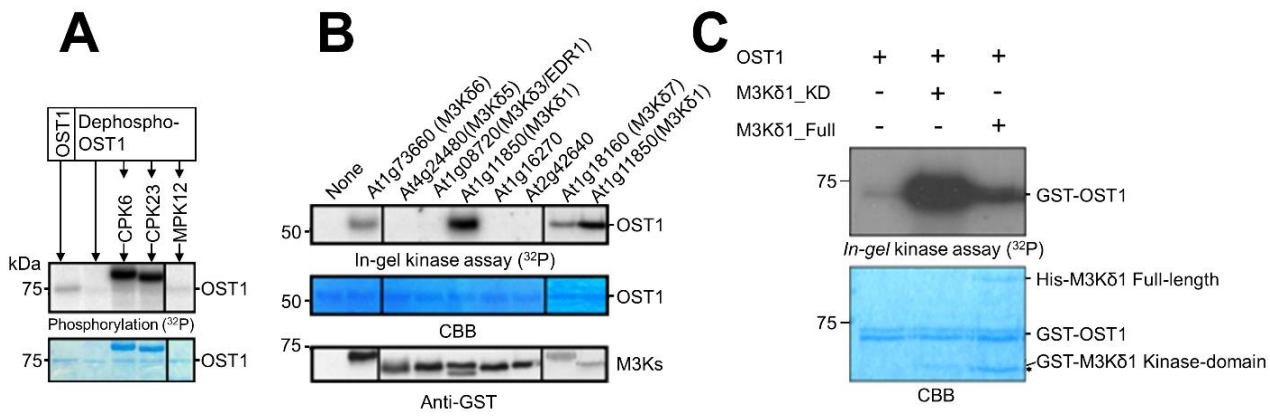

Figure 1. Results indicate M3Ks are essential for reactivation of OST1/SnRK2.6 after dephosphorylation by protein phosphatase in vitro. These results were conducted and reported by Dr. Yohei Takahashi. (source: Takahashi et al., 2020).

(a) In vitro phosphorylation assay de-phosphorylated GST-OST1/SnRK2.6 protein kinase by protein phosphatase cannot reactivate itself. **(b)** In-gel kinase assay result of dephosphorylating recombinant His-OST1/SnRK2.6 protein with the kinase domain of seven M3Ks gene to compare OST1/SnRK2.6 activation activities. **(c)** In-gel kinase assay measured OST1/SnRK2.6 kinase activity after incubating with His-M3Kδ1 Full-length or GST-M3Kδ1 kinase domain for 30 mins in the presence of ATP. (figure source: Takahashi et al., 2020.).

1.2 Overexpression and Inducible construct of M3Ks genes.

Previous results showed that when using CRISPR Cas9 to generate triple mutant knock-out M3Kδ1/6/7 mutants in T-DNA background plants, the plants showed a reduced ABA-sensitive phenotype. The MAPKKK proteins function as an activator by phosphorylating SnRK2 after PYR/PYL/RCAR binds to PP2C in the ABA transduction pathway (Takahashi et al., 2020). This result raises the possibility that by generating an overexpression line of MAPKKKs protein, the phosphorylation of OST1/SnRK2 activities can be increased. As a result, hyper-sensitive ABA responses might be obtained. Therefore, we sought to generate transgenic plants that contain the over-expression of the M3Kδ1/6/7 under the control of the strong guard cell (*pGC1*) promoter (Yang et al., 2008) in the respective Columbia (wild type) background to explore the expression of this gene family. The *pGC1* promoter was developed in our laboratory and has been shown in the past to promote strong expression in guard cells (Yang et al., 2008). The overexpression lines within m3kδ1, m3kδ6 or m3kδ7 were generated using Gateway Cloning technology. First, we used PCR amplification to obtain the gene of interest using Phusion DNA Polymerase, the primer sets sequences were included in Table 1. Such primers were designed to include the *att sites* which are specific for Gateway Cloning technology. The expected DNA fragment lengths were included in Table 2. The gene of interests were cloned into plant compatible vectors with *pGC1* promoter and the green fluorescence protein (*GFP*) fused to the downstream of the gene (Figure 2). The constructs were confirmed through colony PCR using gene-specific binding primers and visualized on 1% agarose gel. Sequencing was done via Retrogen Inc using M13 forward and reverse primers; primer sequences are attached in Table 1. After *Agrobacterium* transformation, seeds of the T1 generation were collected and selected on ½ MS plates containing the antibiotic BASTA. A total of 53 independent transgenic lines have been identified in this antibiotic screening. The germinated

seedlings were transferred onto the sterilized soil to grow for 4 weeks. Plants genomic DNA were isolated, two independent lines confirmed via PCR to contain the mutation in the same generation. Additionally, plants were then being reselected under a confocal microscope to identify the green fluorescent protein (*GFP*) in guard cells. Unfortunately, GFP fluorescence was not detected in the guard cell overexpression transgenic lines, and only guard cell autofluorescence was obtained (Figure 3a-b). It had been proposed that one potential explanation for this is that the expression levels were too low. Candidate #4 was used for further analyses.

Table 1. Primer sequences that are used for Cloning overexpression vectors via Gateway Cloning system.

	Primer Sequences	Tm(°C)
M3kδ1 Kinase Domain Forward	5'-ggggacaagtttgtacaaaaaagcaggctccatg GAAGCTGCAAAGTGGGAAATTATG	55
M3kδ1 Full Length Forward	5'-ggggacaagtttgtacaaaaaagcaggctcc ATGTCGAAGATGAAGCATCTTCTAC	55
M3kδ1 Reverse	5'-ggggaccactttgtacaagaaagctgggtc CATCAAACTTTCACTCGTATTCGC	55
M3kδ6 Kinase Domain Forward	5'-ggggacaagtttgtacaaaaaagcaggctccatg ATGTATCTGACTGTGAGATTTTGTGG	55
M3kδ6 Full Length Forward	5'-ggggacaagtttgtacaaaaaagcaggctcc ATGAAAGTAAAAGAAGAAACTTTGAAGAATTTG	55
M3kδ6 Reverse	5'-ggggaccactttgtacaagaaagctgggtc ATCCTTTTGTTCATGTTCAGTTGG	54
M3kδ7 Kinase Domain Forward	5'-ggggacaagtttgtacaaaaaagcaggctccatg GCTGCAATTGATGATGTAGCTG	54
M3kδ7 Full Length Forward	5'-ggggacaagtttgtacaaaaaagcaggctcc ATGAAGATGAACATGAAGAAATTTCTCAAAAAG	56
M3kδ7 Reverse	5'-ggggaccactttgtacaagaaagctgggtc TTGTTCCTGCTCATCAGTGG	54
M13 Forward	5′-GTAAAACGACGGCCAG	51
M13 Reverse	5′-CAGGAAACAGCTATGAC	47

Table 2. Expected DNA fragment length after PCR.

	Expect DNA Length (bp)
M3kδ1 Kinase Domain	852
M3kδ1 Full Length	2643
M3kδ6 Kinase Domain	884
M3kδ6 Full Length	3090
M3kδ7 Kinase Domain	882
M3kδ7 Full Length	3009

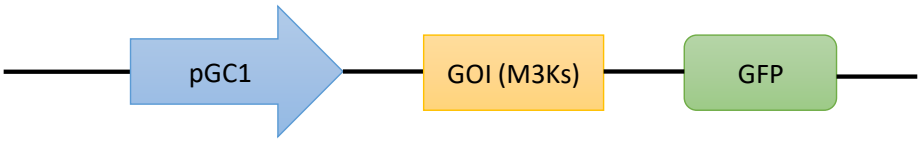

Figure 2. Overexpression construct of *Raf-like MAPKK-kinases (M3Ks)* expressed in Guard cells.

The gene of interest (M3Ks) is under the control of a strong guard cell promoter (*pGC1*) upstream of the gene of interest. Green fluorescence protein (*GFP*) is at the 3' end of the gene for markers and able to be detected under a confocal microscope.

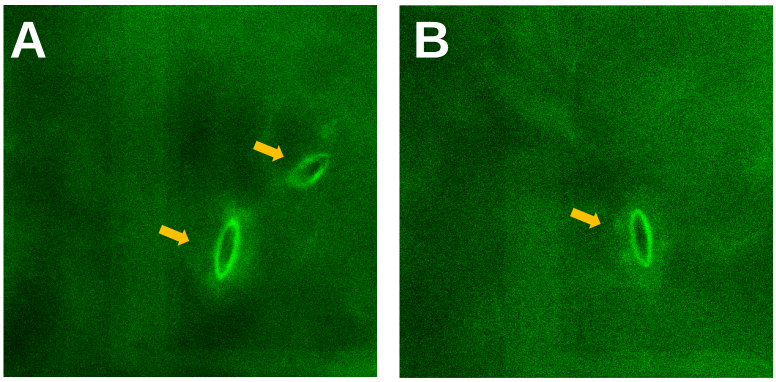

Figure 3. Confocal Microscopy Analyses of Guard Cell overexpression expression of M3Kδ1 Kinase Domain.

(A) Control fluorescence of two stomata as indicated by arrows of wild type (Columbia). **(B)** Overexpression pGC1:M3Kδ1 Kinase Domain: GFP transgenic line do not show clear GFP fluorescence (arrow) under 40X confocal microscopy; 53 independent lines were observed.

To improve our chances of identifying different mutant phenotypes and gain a more in-depth understanding of the function of the M3Ks, inducible m3kδ1 expression in guard cells was also tested using an EtOH inducible promoter (M.X. Cadddick et al., 1997) via USER cloning system (Figure 4). Under the ethanol inducible version, the gene of interest would only be expressed when ethanol is present and binds to the alcohol receptor (*alcR*). After obtaining the overexpression construct vectors using Gateway Cloning (Figure 2), specific binding forward primers were designed to target the 5' of the gene of interest and reverse primer anneal to the 3' end of the *GFP* tag (Table 3). The inducible constructs were confirmed by sequencing from Retrogen Inc. and transformed into *Arabidopsis thaliana* via the *Agrobacterium* transformation technique. Plants were grown in the growth room condition with 16 hours light and 8 hours dark. Seeds selection was performed on over 400 lines using Hygromycin antibiotic screening; germinated seeds were transferred onto the soil to grow. Since the vector construct had the green fluorescent protein tag (*GFP*) at the C-terminus, the confocal microscope was used to confirm the transformation under the GFP filter using the abaxial side of the leaves. After incubating the leaves of the EtOH inducible phenotype onto 2% ethanol, one line displayed consistently strong GFP fluorescence in guard cells, but not H_2O control treatment leaves (Figure 5a-b).

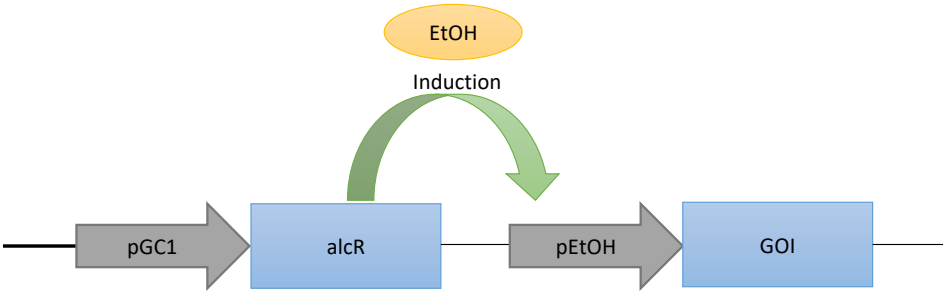

Figure 4. Development of inducible vector constructs via EtOH-induced expression in guard cells.

The vector was created with the help of Dr. Yohei Takahashi. In the presence of ethanol, alcR proteins will bind to pEtOH in which gene expression will be induced. The pEtOH promoter function is under the control of the guard cell promoter *pGC1*. The gene of interest (GOI) can be cloned into the downstream region of the pEtOH promoter by USER Cloning with a select choice of the tag of interest.

Table 3. Primer sequences that are used for Cloning inducible vectors via the USER cloning system.

	Primer Sequences	Tm(°C)
M3kδ1 Kinase Domain Forward	5'-ggcttaauATGGAAGCTGCAAAGTGGGAAATTATG	56
M3kδ1 Full Length Forward	5'-ggcttaauATGTCGAAGATGAAGCATCTTC	54
M3kδ6 Kinase Domain Forward	5'-ggcttaauGATGTATCTGACTGTGAG	52
M3kδ6 Full Length Forward	5'-ggtttaauATGAAAGTAAAAGAAGAAACTTTGA	56
M3kδ7 Kinase Domain Forward	5'-ggcttaauGCTGCAATTGATGATGTAG	52
M3kδ7 Full Length Forward	5'-ggcttaauATGAAGATGAACATGAAGAAATTTC	53
GFP Reverse Primer	5'-ggtttaauTTATTTGTATAGTTCATCC	52
At2g45530_user.F52	5'-ggcttaauATGCAAAGTAGCTCCAGC	52
At2g45530_user.RP54	5'-ggtttaauCAAGGCAGGTGGGTAC	54

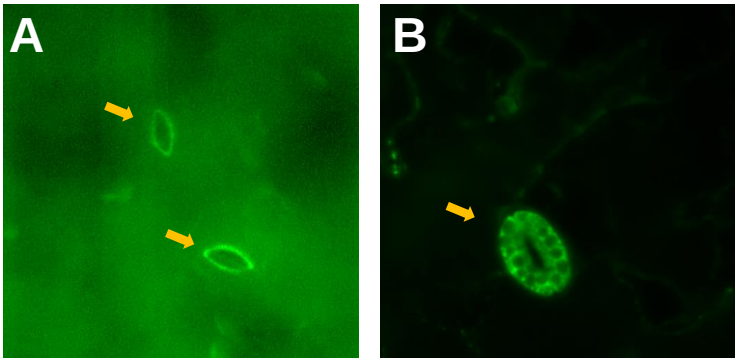

Figure 5. Confocal Results of Guard Cell Inducible expression of M3Kδ1 Kinase Domain.

(A) Control autofluorescence of two stomata (arrows) in H2O treatment after 2 hours incubation of pEtOH-pGC1:M3Kδ1 Kinase Domain: GFP transgenic line. **(B)** Alcohol Inducible pEtOH-pGC1: M3Kδ1 Kinase Domain: GFP guard cell expression after 2 hours treatment with 2% ethanol (arrow).

Table 4. Vector constructs and antibiotic use, continued.

BP Reaction:

Vectors	Insertion	Antibiotics
pDONR211	M3kδ1 Kinase Domain	Kanamycin/ chloramphenicol
pDONR211	M3kδ1 Full Length	Kanamycin/ chloramphenicol
pDONR211	M3kδ6 Kinase Domain	Kanamycin/ chloramphenicol
pDONR211	M3kδ6 Full Length	Kanamycin/ chloramphenicol
pDONR211	M3kδ7 Kinase Domain	Kanamycin/ chloramphenicol
pDONR211	M3kδ7 Full Length	Kanamycin/ chloramphenicol

LR Reaction:

Vectors	Construct	Antibiotics
pBASTA	pGC1- M3kδ1 Kinase Domain- GFP	Spectinomycin/ chloramphenicol
pBASTA	pGC1- M3kδ1 Full Length- GFP	Spectinomycin/ chloramphenicol
pBASTA	pGC1- M3kδ6 Kinase Domain- GFP	Spectinomycin/ chloramphenicol
pBASTA	pGC1- M3kδ6 Full Length- GFP	Spectinomycin/ chloramphenicol
pBASTA	pGC1- M3kδ7 Kinase Domain- GFP	Spectinomycin/ chloramphenicol
pBASTA	pGC1- M3kδ7 Full Length- GFP	Spectinomycin/ chloramphenicol

Table 4. Continued

USERs Cloning: EtOH-induced expression.

Vectors	**Construct**	**Antibiotics**
pBASTA	pEtOH- M3kδ1 Kinase Domain- GFP	Kanamycin 50
pBASTA	pGC1- M3kδ1 Full Length- GFP	Kanamycin 50
pBASTA	pGC1- M3kδ6 Kinase Domain- GFP	Kanamycin 50
pBASTA	pGC1- M3kδ6 Full Length- GFP	Kanamycin 50
pBASTA	pGC1- M3kδ7 Kinase Domain- GFP	Kanamycin 50
pBASTA	pGC1- M3kδ7 Full Length- GFP	Kanamycin 50

Table 5. Multisite Gateway plasmid used from Schroeder's lab clone stock (as E. coli glycerol stocks) BOX #70.

Ref #	Stock Type	Clone Name	General Description	Vector	Strain	Antibiotic
5504	Glycerol Stock	pB7m34GW,0	Binary vector used for plant expression. Carry a Basta resistance cassette. Multisite GW compatible	pB7m34GW,0	DB3.1	Spec100; Cm25
5509	Glycerol Stock	pDONR221 P1P2 (p221)	Binary vector. Multisite GW compatible	pDONR221 P1P2 (p221)	DB3.1	Kana 50; Cm 25
5521	Glycerol Stock	pGC1 / p5'	Promoter GC1 in a pDONR P4P1r (p5')	pDONR221 P4P1r (p5')	TOP10	Kana 50
5534	Glycerol Stock	GFP w/ STOP / p3'	GFP w/ STOP in a pDONR221. for Cter fusions	pDONR221 P2rP3 (p3')	TOP10	Kana 50

1.3 Thermal imaging of the transgenic in Arabidopsis thaliana for stomatal responses.

A forward genetic screen was used to compare the phenotype between mutagenized populations and the wild type (control). The study of stomata is useful to study to potentially improve crop production in response to abiotic stress, photosynthetic yield, and water usage (Asseng et al., 2015). In dicot, stomata are made up of two kidney-shaped guard cells that work together with subsidiary cells to control stomatal movement (Woodward et al., 2003). The pair of guard cells present in the epidermis of leaves function to regulate gas exchange and evapotranspiration. Perceived stressful conditions such as exposing plants to ABA or ethanol could lead to the closure of stomatal pores on the surface of the leaves. Plants close the stomatal pore to prevent excessive water loss through evapotranspiration, which will raise the canopy leaf temperatures and can be observed through thermal imaging. Thermography is a useful method to observe the differences between canopy leaf temperatures of mutant plants and wild-type plants under different stress conditions based on the concept of stomatal response impairment. The technique provides the indirect potential differences in stomatal activities between transgenic lines and wild type without harming the plants and their life cycle (Leinonen et al., 2004; Merlot et al., 2002; Ruiz et al., 2016). Stomatal closing reduces evapotranspiration and therefore leaves become warmer upon stress-induced stomatal closing. If mutants are identified to have cooler leaves temperatures under stressful conditions that are shown to cause stomatal closure and cause leaf warming, the mutants might contain causative mutations in genes that play important roles in the CO_2 or ABA signaling pathways that mediate stomatal closing (Wang et al., 2004). Here in this study, *Columbia 0* was used as a control as all mutagenized lines are within the Columbia background which has been previously sequenced, and High Leaf Temperature (*ht1-2*) mutant plants were used as the positive control. The transgenic overexpression and inducible expression

of the *M3K* genes were transformed in the Columbia (*Col*) background. Plants were grown under 16 hours light and 8 hours dark for 4 weeks in a growth chamber with ambient CO_2 level at 400 ppm. Thermal imaging of overexpression and inducible transgenic mutagenized plants were taken before being sprayed with 20 μM ABA (overexpression) or 5% ethanol (inducible). Plants were then transferred into an ambient CO_2 chamber (400 ppm) for 3 hours and thermal imaging was taken. The thermal images show the transgenic lines of *M3Kδ1* expressed in guard cells are hotter than wildtype (Col-0), and similar temperature to the known High Leaf temperature (*ht1-2*) mutants after exposure to the same conditions (Figure 6, 7).

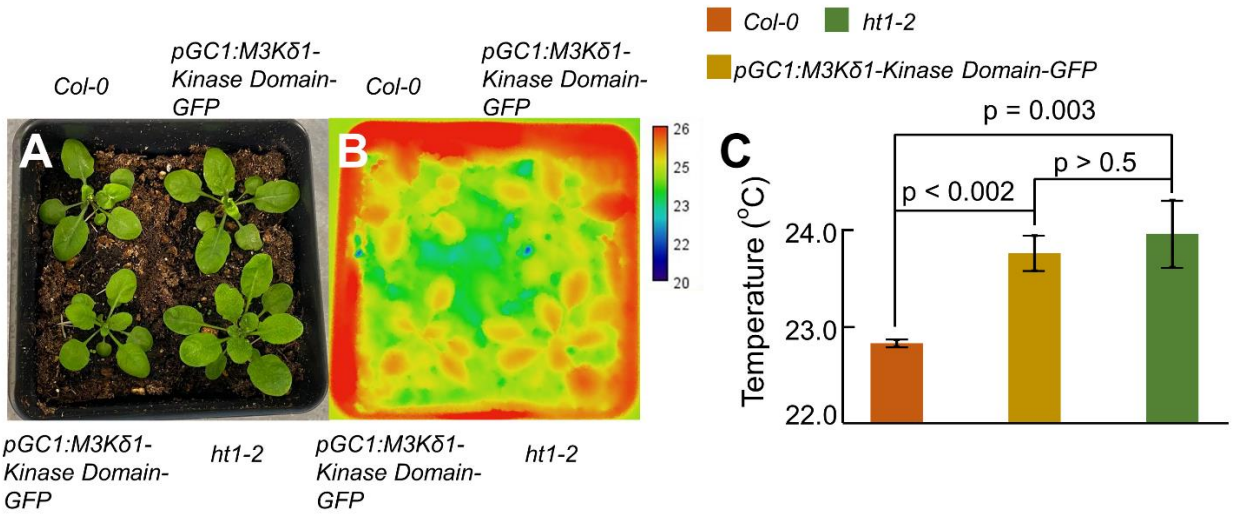

Figure 6. Leaf temperatures of *Col*, *pGC1-D1KD-GFP*, and *ht1-2* were measured by thermal imaging.

(a) The brightfield image shows four plants. **(b)** Thermal images were taken 3 hours after plants were sprayed with 20 μM ABA. **(c)** Average leaf temperatures were measured by using Fiji software which shows the temperature increase for the transgenic line compare to the *ht1-2* mutant (n = 3 experiments). Data represent mean ± s.e.m and analyzed by one-way ANOVA test.

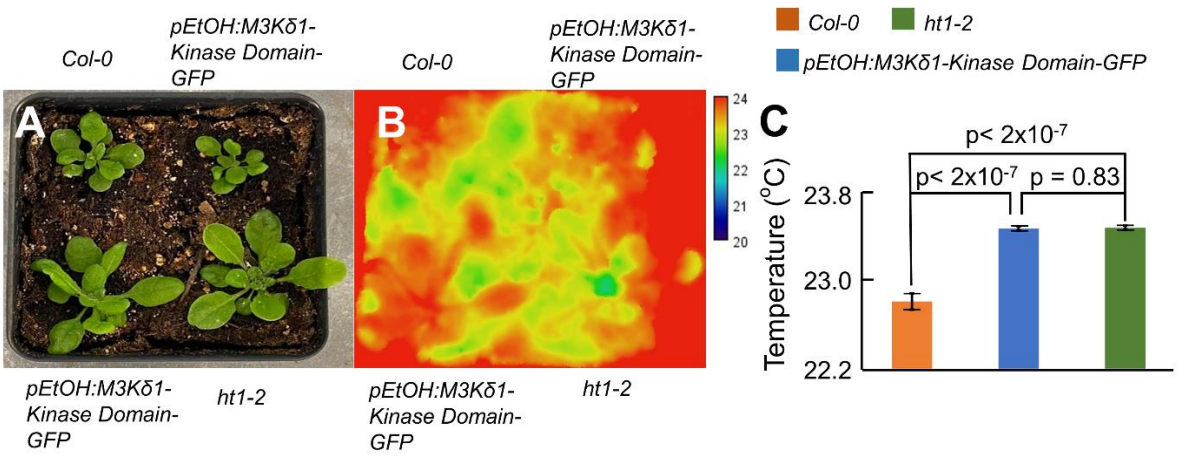

Figure 7. Leaf temperatures of *Col*, *pEtOH-D1KD-GFP*, and *ht1-2* were measured by thermal imaging.

(a) The brightfield image shows four plants. **(b)** Leaf temperatures of plants were recorded 3 hours after they were sprayed with 5 % ethanol. **(c)** Average leaf temperatures were measured by using Fiji software which shows the temperature increase for the transgenic line compare to the *ht1-2* mutant (n = 3 experiments). Data represent mean ± s.e.m and analyzed by one-way ANOVA test.

1.4 Characterization of the ethanol inducible version of Raf-Kδ1 truncated constitutively active.

While the overexpression and inducible candidates show higher leaf temperatures upon contact with ABA (overexpression) and ethanol (inducible), there is still a lot of unknown about the stomatal response. Plants are known to rapidly open and close stomata in response to environmental changes (Hsu et al., 2018). To further analyze the difference in stomatal phenotype, intact leaf gas exchange was performed on the transgenic line (EtOH-inducible), wildtype (*Col-0*), and *ht1-2* to the identification of possible stomatal impairment. Stomatal conductance can provide real-time analysis of the mutant phenotype's ability to control stomatal apertures. All plants were sprayed with 5% ethanol and incubated in a growth chamber with ambient CO_2. After two hours, using the intact leaf gas exchange system, plants were allowed to equilibrate for 1 hour and then exposed to ambient CO_2 for another hour to observe stomatal conductance. Upon obtaining the results, the ethanol inducible version of Raf-Kδ1 truncated constitutively active (CA) constructs showed a lower stomatal conductance than WT and similar to the stomatal conductance of positive control *ht1-2* plants (Figure 8). The preliminary data suggest that upon increasing the expression of the M3Ks gene, the transgenic plants are able to close stomata more than the wild type, which potentially allows plants to display higher water use efficiency.

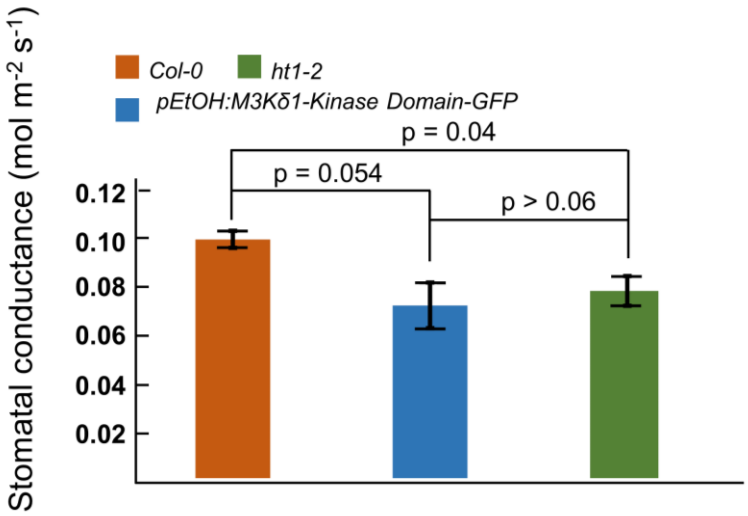

Figure 8. **Characterization of ethanol inducible Rafδ1-truncated inactive**.

The average stomatal conductance analysis using *Col-0*, *ht1-2*, and *pEtOH-M3K Delta 1 Kinase Domain-GFP* in response to exposing with 5% Ethanol. Light strength: 150 µmol.m^{-2}.s^{-1} with 10% blue light; Ambient CO_2: 400 ppm; data recorded for 60 mins; n= 4 for each genotype. Data represent error bars denote mean ± s.e.m and analyzed by one-way ANOVA test.

1.5 M3Kδ6 and At2g45530 interact in planta.

Through data mining of the Membrane-based Interactome Database (MIND), At2g45530- the Ring U-box protein showed a high interaction score with delta 7 (Figure 9) (Jones et al., 2014). M3Kδ6 and M3Kδ7 have been identified as key components for the ABA signaling pathway (Takahashi et al., 2020), making it an excellent candidate as an interactor with the U box gene. To find potential interactions of M3Kδ6/M3Kδ7 involved in the ABA signaling pathway (Takahashi et al., 2020), bimolecular fluorescence complementation assays (BiFC) (Waadt et al., 2008) were used to investigate the protein-protein interactions among At1g73660/At1g11860 (m3kδ6/m3kδ7) and At2g45530 (U-Box E3). BiFC analyses can determine transient interactions in plant cells. The technique relies upon the binding form of a yellow fluorescence protein complex (YFP) by dividing into two non-fluorescent fragments (nYFP: N-terminal YFP fragment and cYFP: C-terminal YFP fragment); when two protein fuse the fragments together, the fluorescent can be obtained. The fluorescent can be used to quantitatively presume how strongly the protein interaction as well as any detection of weak and/or transient protein interaction (Kerppola et al., 2006). In these experiments, m3kδ6/m3kδ7 were fused with N-terminal fragments of YFP (N-m3kδ6/m3kδ7) and co-expressed in leaves of *N. benthamiana* epidermal cells with At2g45530 that fused with C-terminal of YFP. After 3 days, the subcellular localization and YFP emission of the recombinant complex were analyzed by confocal microscopy. The complex of N-YFP-m3kδ7 and At2g45530-C-YFP showed no clear interaction by the absence of fluorescence when the subcellular localization and YFP emission complexes were observed under confocal microscopy (Figure 9e). Conversely, quantitative BiFC experiments provide evidence that m3kδ6 binds to At2g45530 in plant cells under the confocal microscope (Figure 9c-d). Preliminary data indicate that m3kδ6 might exhibit

interaction with At2g45530.

A

	Score	Name	Type
AT2G45530	***	RING/U-box superfamily protein	E3 ligase
AT4G10850	*	SWEET7	Transporter
AT2G01420	*	PIN4	Transporter

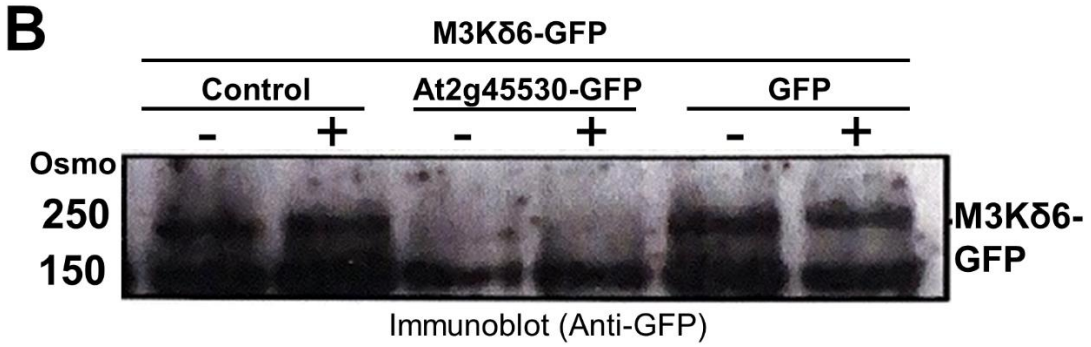

Figure 9. M3Kδ6 and M3Kδ7 might interact with RING/U-box E3 ubiquitin ligase. Data from Dr. Yohei Takahashi, unpublished.

(A) M3Kδ7 was searched in Membrane-based Interactome Database (MIND) and showed interaction with RING/U-box E3 ubiquitin ligase. **(B)** M3Kδ6-GFP was transiently expressed in mesophyll cell protoplasts with At2g45530-GFP or GFP using the PEG mediated method. M3Kδ6-GFP proteins were detected by immunoblot analysis using a GFP antibody.

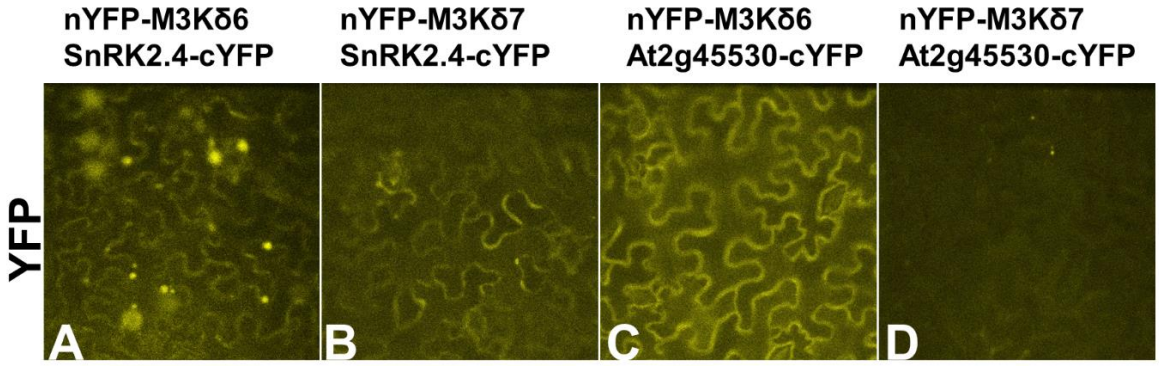

Figure 10. M3Kδ6 may interact with At2g45530 in BiFC experiments in plant cells.

(A-D) BiFC analyses of nYFP-M3Kδ6 or nYFP-M3Kδ7 with At2g45530-cYFP infiltrated in 6-week-old Nicotiana benthamiana leaves. nYFP-M3Kδ6 or nYFP-M3Kδ7 with SnRK2.4-cYFP were used as the positive control (n=4).

Discussion

In *Arabidopsis*, the activation of OST1/SnRK2 protein kinase is required for the ABA signaling pathway (Cutler et al., 2010; Fujii et al., 2007; Raghavendra et al., 2010; Zhu, 2016). Recent research in our laboratory identified M3Ks genes as being needed for OST1/SnRK2 activation (Figure 1) (Takahashi et al., 2020). The phosphorylation of OST1/SnRK2 is required for the ABA response, to further examine the function of *M3Ks* genes, the overexpression and inducible transgenic plants have been successfully generated to compare expression with wild-type plants (Figures 2 and 4). Several questions with these transgenic plants were explored. In the present study, the first tool for the inducible expression of genes in guard cells was developed. Applying this approach suggests that M3Kδ1 over-expression reduces stomatal conductance leading to warmer leaf temperatures. These datas further suggest a physiological effect of M3Ks over-expression on guard cell signal transduction.

With support from Dr. Yohei Takahashi, not only was a stable mutant line of M3K genes created but the present result reports the development of a first guard cell inducible vector construct tool which will be helpful for future experiments. The construct demonstrates the potential guard cell inducible manipulation in transgenic plants. Upon induction of ethanol, the mutant phenotype displays strong guard cell fluorescence and can be detected under confocal microscopy. This is important since, without the transcriptional inducer (ethanol) with the receptor (alcR), the transgenic plants grow normally until induced by ethanol treatment. Under normal conditions, the levels of protein expression in plants will remain unchanged compared with wildtype controls; only when the transcriptional inducer is activated will transgene expression be induced. The inducible system provides a tool for easy identification of genes that participate in the pathway of interest in plants.

Experimental evidence for guard cell-targeted gene overexpression and induction system have not been reported in *planta*. Through the initial antibiotic screen, over 300 independent candidate transgenic lines of overexpression in the F1 generation were screened, and 53 lines survived the BASTA selection. Upon completion of the initial screen, confocal microscopy results indicated only autofluorescence was observed, similar to the wildtype control. To identify whether the plants contained the construct, plant genomic DNA was isolated from the 53 lines selected in the initial screen. In the T1 generation, resulting in 2 mutants were shown to contain the overexpression construct. One possible explanation for this observation might be due to the low expression of the gene upon transform into plants. The two mutants that were identified in the tertiary screen were used for further characterization. Unlike the overexpression version, the ethanol inducible guard cell expression using EtOH inducible promoter showed a strong GFP fluorescence in the secondary screen using the confocal microscope in F1 and again in the F2 generation. After confirming the generated mutant phenotype of M3Ks overexpression and induction plants, several questions were explored:

1. Is there a difference between M3Ks transgenic plants and *Col-0* wild-type plants?
2. Can ABA or EtOH treatment affect stomatal activity for the M3Ks transgenic plants and *Col-0* wild-type plants?

The CO_2 and ABA signaling pathways mediate stomatal responses and control leaf temperature (Merlot et al., 2002; Hsu et al., 2018; Takahashi et al., 2020). The *Arabidopsis ht1-2* mutant was shown to have warmer leaves temperature even without stress environments (Hashimoto et al., 2006, Natrisiva et al., 2015). During the first infrared thermal imaging screening, the transgenic lines of both overexpression and induction plants displayed more subtle temperature differences. Leaf temperatures of both versions of M3Kδ1 genes show an increase in

temperature similar to the known “High Temperature 1” (*ht1-2*) mutant, which indicates stomatal closure (Figure 6, 7). One possible explanation for the difference in leaf temperature is believed to be due to the increase of phosphorylation of OST1/SnRK2 by the overexpression of M3K protein. By doing so, the transgenic line plants were apparently able to close stomata more than those of wild-type controls. Another explanation that is considered to explain the phenotype could be the transgenic line cause by stomatal development. Plants contain fewer or smaller stomata on the surface, which could potentially decrease the gas exchange rate through their stomatal pores, which also decreases the rates of evapotranspiration, as the result, higher canopy leaf temperature (Vráblová et al., 2017). However, transgenic line plants were appearing similar to wild type, so the phenotype is unlikely due to the stomatal development. Further thermal imaging screens in the overexpression and inducible phenotypes, stomatal density, and index analyses will be needed to determine conclusively how the inducible lines affect stomatal responses.

Following thermal imaging, intact leaf gas exchange was performed. It was observed that after exposure to 5% ethanol under ambient CO_2, stomatal conductance was lower than wild-type control and was reduced almost the same stomatal conductance as the *ht1-2* mutant positive control after extended periods. All plants were grown in similar conditions and exposed to the same 5% ethanol for 3 hours. An explanation for these observations was the hypothesis that perhaps the inducible phenotype is due to increased phosphorylation of OST1/SnRK2, which leads to a lower stomatal conductance. However, further investigation such as repeat thermal imaging, measuring stomatal pore length, aperture, density, and subsidiary cell size will be needed to understand the mechanisms mediating M3K over-expression induced stomatal conductance reduction and any effects on SnRK2 protein kinase activity.

Materials and Methods:

1.1 Plant growth conditions and selection assay.

Seeds were sterilized in 15 minutes incubation in a solution containing 20% bleach and 0.5% Tween 20 followed by two times washing steps with distilled water. The seeds were then suspended in 0.05% agar and sown on ½ Murashige and Skooge (Sigma) plates with a pH of 5.6. After three days of stratification at 4^{o}C, the plates then being moved to the growth room with the 16 hours light and 8 hours dark conditions. After 7 days on the plates, germinated seedlings were transferred into pots of sterilized soil.

For the seed's selection assay, seeds were incubated in a solution containing 70% ethanol and 0.05% SDS for 20 minutes and washed with 100% ethanol two times. The sterilized seeds were then sewn onto the selection plates (BASTA/ Hygromycin). The plates were introduced into stratification at 4^{o}C in the dark for four days, these plates were then transferred to the growth room in long-day condition. After 5 days old, germinated seedlings were transferred ½ MS plates (pH 5.6). After 5 days, the germinated seedlings were transferred to sterilize soil to further growth.

1.2 Cloning of M3Ks Overexpression using Gateway Multisite Cloning Technique.

The gene of interest was obtained via PCR using Phusion DNA Polymerase. The regions were amplified using PCR on genomic DNA via right- and left- primers with the appropriate flanking site for Gateway Cloning (GW). The primers sequence is provided in Table 1 in the. The PCR products were then run on the 1% Agarose Gel via gel electrophoresis for 10-15 minutes at ~85 mV (Table 1). The gel bands were then purified using NucleoSpin Gel and PCR Clean up Kit (Thermo Scientific). The plasmid vectors were isolated using Plasmid Miniprep Kit (Thermo Scientific). The technique that was used to clone the gene of interest was Gateway Multisite Cloning (Invitrogen). The competent cell strain used was DB3.1.

The BP reactions contained 1 μL of the gene of interest (GOI), 1 μL of destination vector, 6 μL of TE buffer (pH 8.0), and 2 μL of BP Clonase II enzyme. The reaction mixtures were incubated overnight at room temperature, then transformed into *Escherichia coli* through the heat-shock method the following day. The competent cells were thawed on ice for 2 minutes, 10 μL of the BP reaction DNA products were used in transformation, followed by incubation on ice for 30 minutes. The competent cells were heat-shocked at 42^{o}C for 50 seconds, then 500 μL of sterilized liquid LB was added and incubated on a shaker at 37^{o}C for 45 minutes. The incubated cells were plated onto selected 50 μg/mL Kanamycin media and incubated overnight at 37^{o}C. A single colony was picked and a plasmid of transformants was isolated using Plasmid Miniprep Kit. BP reactions were confirmed by sequencing using M13 Forward and Reverse primers (Table 1).

Using the DNA from the BP reaction, LR reactions were performed for multisite cloning. The LR reaction mixtures contained 1 μL of the desired promoter, BP product of the gene of interest, *GFP* tag, destination vector, 8 μL of TE buffer (pH 8.0), and 1 μL of LR Clonase II enzyme. The reactions were incubated overnight at room temperature. The mixtures were then transformed into *Escherichia coli* by the heat shock method and DNA was isolated using the Plasmid Miniprep Kit described previously with the selection of 100 μg/mL Spectinomycin antibiotic. LR reactions were again confirmed by sequencing using M13 Forward and Reverse primers (Table 1). The vector constructs were then transformed into *Agrobacterium tumefaciens* with 100 μg/mL Spectinomycin and 50 μg/mL Gentamicin antibiotic.

1.3 Plant material and Agrobacterium tumefaciens transformation.

Transformation of *Arabidopsis thaliana* was performed by the floral dipping method via *Agrobacterium tumefaciens* strain *GV3101*. The vectors used were described previously with guard cell promoter (*GC1*) on the 5' UTR and *GFP* tag on the 3' backbone. The *A. tumefacien* cells were thawed at room temperature for 3 minutes. 5 μL of positively constructed DNA plasmids were then added to the competent cells and immediately submerged liquid Nitrogen to snap freeze cells. The cells were incubated at 37^{o}C for 10 minutes and 400 μL of liquid LB added before incubating on the shaker at 28^{o}C for 2 hours. The cells were transferred onto 100 μg/mL Spectinomycin and 50 μg/mL Gentamicin antibiotic and incubate at 28^{o}C for 2 days.

1.4 Arabidopsis thaliana transformation using floral dip transformation.

Wild type *A.thaliana* plants in the Columbia background were grown under long days conditions in separate pots until flowering. A selected colony was picked from Spectinomycin/Gentamicin plates as described previously and bacteria were resuspended in 5 mL of liquid LB with Spectinomycin/Gentamicin, the cultures were incubated on a shaker at 28^{o}C overnight. The next day, the cultures were upscale to a larger volume (200 mL) using sterilized liquid LB with Spectinomycin/Gentamicin. For the following days, the cell cultures were centrifuged at 5000 rpm for 15 minutes, the supernatants were discarded. The cells were resuspended in the solution contained ½ MS, 5% Sucrose, and 0.02% Silwet L-77. Before performing floral dipping, all siliques were removed. Plants were transformed through floral dipping of inflorescences. Each pot was dipped into the solution for 50 seconds two times, a small amount of solution (~300 μL) was added onto the roots to ensure everything was in contact with the solution. The transformed pots were then laid on their sides and covered with aluminum foil for 18 hours. After uncovering the tray, plants were water and grew normally until seeds became mature.

1.5 Thermal Imaging.

Thermal imaging was performed on 5-week-old plants using infra-red FLIR T650sc (FLIR Systems, Inc. Wilsonville, OR 97070 USA); the camera was equipped with a 25^{o} lens. The temperature accuracy ± 0.25^{o} C at room temperature. The plants first spray with either 20 μM ABA or 5% EtOH and placed at ambient CO_2 within the growth room conditions with approximately 400 ppm. Images were taken after 3 hours.

1.6 Licor Gas Exchange Analysis.

Intact leaf stomatal conductance gas exchange was performed on leaves of 5–to 6-week-old plants using portable gas exchange systems (LI-6400XT, LI-COR, Lincoln, NE, USA). For intact leaf ethanol treatments, plants were sprayed with 5% ethanol and placed in a growth chamber at ambient 400 ppm CO_2 for 3 hours. The chamber light source was set at 150 μmol m^{-2} s^{-1} (10% blue light) and temperature at 21 °C was used. Immediately after 3 hours, leaves were equilibrating for 1 hour at 400 ppm, 70% humidity with airflow of 200 μmol s^{-1}. Measurements of steady-state stomatal conductance were recorded for another hour. The data are representative of a minimum of n= 3 with each leaf from independent plants per genotype.

1.7 Subcellular localization and BiFC analyses.

For subcellular localization and BiFC analyses, the coding sequences of At2g45530 were clone into a plant binary vector without stop codon. The expressions are under the control of the *pUBQ10* promoter (At4g055310; Norris et al., 1993; Krebs et al., 2011). The coding sequences were cloned into Kan IISPYCE(MR) and hygII-SPYNE(R) plasmids (Waadt et al., 2008). Plasmids were transformed into *Agrobacterium tumefaciens* (GV3101) (pMP90; Koncz and Schell, 1986) and co-infiltrated with the silencing suppressor p19 (Voinnet et al., 2003) into leaves of 6-week-old *Nicotiana benthamian* plants (Waadt et al., 2013). Subcellular localization and BiFC analyses were performed by confocal microscopy using Nikon Eclipse TE2000-U confocal microscope. Images were acquired using Metamorph software version 7.7.7.0 to obtain identical settings (exposure time and gain). Images for quantitative BiFC analyses taken using the 20X objective lens and analyzed, processed in ImageJ (Schindelin et al., 2012; Waadt et al., 2017). Three independent experiments were conducted where 3 leaves were analyzed for each combination.

Chapter 2 Characterize MPK4 and HT1 genes in the CO_2 sensing signaling pathway.

Another stress-inducing stimulus stomatal closure is the presence of high CO_2 in response to drought stress. Through the stomatal pores, plants can conduct gas exchange with their surroundings which allows them to take up CO_2 and in exchange, release O_2 and water vapor. To further identify key components that participate in the regulation of the CO_2 signaling pathway, characterization of mutants identified in a screen of CO_2 insensitive *Arabidopsis thaliana* has been pursued. Even though the CO_2 signaling pathway has been widely investigated, not all components have yet to be identified. Therefore, to identify key elements involved in the pathway, protein binding assays were conducted to find the unknown component of the CO_2 signaling pathway. The mitogen-activated protein (MAP) kinase MPK4/12 is known to participate in the early event of CO_2 stomatal closure. In chapter 2, recombinant MPK4/12 and HT1 protein were being purified from *E.coli* with appropriate GST and His tag to investigate the CO_2 signaling. Using the produced recombinant protein, further biochemical experiments will be performed to study MPK4/12, HT1, and CBC1 protein role in activation of guard cell S-type anion channel in response. Further investigation will be needed to characterize the CO_2 sensing signaling pathway to predict the effect of increasing CO_2 to crop productivity and yields.

Introduction:

Through the last 800,000 years, the CO_2 concentrations in the earth's atmosphere have remained below 300 ppm; however, ever since the industrial revolution, the concentrations have spiked significantly. CO_2 levels in the earth's atmosphere are in the uptrend rising every day. Elevated CO_2 is one of the major contributors to high climate change, frequency/ duration/ intensity of heatwaves around the world (Headquarters, 2014; Stocker et al., 2013). Global atmospheric CO_2 concentration in the atmosphere is at the highest since humans inhabited the planet (411.72 ppm) (https://sioweb.ucsd.edu/programs/keelingcurve/); further studies are requiring understanding the major contributors of the CO_2 signaling pathway. Upon the environmental changes, plants need to adapt to their surroundings to maximize their growth. One of the ways to study plants is through stomatal activities.

Stomata play important roles in the survival of plants by controlling the pores to prevent plants from dry out. On the surface of the leaves, the stomata pores facilitate the gas exchange to adapt to their physiology (Sherrard & Meherali, 2006). Each stomate makes up of a pair of guard cells that control opening and closing by increasing or reduce the turgor pressure (Willmer et al., 1996). The stomatal open by the movement of water and ions across the guard cell in which increase the turgor pressure (Kollist et al., 2014). Low CO_2 is a signal to cause stomatal to be open to increase the influx of CO_2 for assimilation. When high CO_2 was detected, plants showed a decrease in stomatal conductance which leads to a decrease in photosynthetic rates (Kanemoto et al., 2009; Xu et al., 2016).

Stomatal closure is initiated as CO_2 enters the guard cells which triggering downstream signaling events (Willmer et al., 1996; Kollist et al., 2014; Kanomoto et al., 2009). The CO_2 signal cascade enters the cells via an aquaporin known as *PIP2*. *PIP2* showed to interact with

Beta Carbonic Anhydrase 4 (*βCA4*) which allows bicarbonate (HCO_3^-) formation to accelerate (C. Wang et al., 2016). Upon the increase of HCO_3^-, Mitogen-Activated Protein Kinase 4/12 (MPK4/12) and High Leaf Temperature 1 (*HT1*) mediate the signal transduction. Previous studies showed that *MAPK4/12* participate and serves as essential regulators in response to high CO_2 which triggers downstream stomatal closing (Toldsepp et al., 2018, Hiyama et al., 2017). *HT1* serves as a negative regulator of stomatal; the protein kinase phosphorylation the downstream activity of Convergence of blue light and CO_2 (*CBC1/2*) in which will trigger stomatal opening by inhibiting Slow Anion Channel Associated 1 (*SLAC1*) (Hiyama et al., 2017). *SLAC1* is functioned as a bicarbonate sensor responding to CO_2/ HCO_3^- and has been shown to upregulate by Guard Cell Hydrogen Peroxide Resistant 1 (*GHR1*) in which will promote stomatal closure upon being activated (Zhang et al., 2018). For stomatal to close, *SLAC1* opens the channel which allows anions to flow out of the cells to depolarize the membrane, which triggers the K+ channel to open and efflux of K+ ion which causes the decrease in turgor pressure inside the cell (Zhang et al., 2018). Genetic evidence will be needed to demonstrate the CO_2 sensing and signaling by MPK4/12-HT1 CBC cascade. In order to reveal functions of MPK4/12, CBC1/2, HT1 protein kinases, biochemical and genetic research such as protein interaction, kinase activation, HCO_3^- dependent regulation is needed. To conduct these experiments, a large amount of recombinant protein is needed; this was done through *E.coli* cells with plasmids of interest and inducing protein expression purification. These proteins have been used in further in vitro experiments to reveal protein functions.

Results and Discussion.

2.1 Protein Purification for binding and structural assay.

The rising of CO_2 in the earth's atmosphere could lead to an increased frequency of heatwaves which is shown to negatively impact the environment, and agriculture, and crop yield (Zheng et al., 2019; Asseng et al., 2015; Wahid et al., 2007). Upon the increase of CO_2 concentration, plants need to develop a mechanism to survive and adapt to the environment. When plants detect the increasing CO_2 concentration, the stomatal pores on the surface of the leaves will be close to avoid excess water loss. Research has demonstrated that in a high CO_2 environment, mitogen-activated protein kinases MPK4 and MPK12 perform an important role in the stomatal response (Jakobson et al., 2016; Horak et al., 2016; Marten et al., 2008; Toldsepp et al., 2018); however, how the proteins participate in the stomatal CO_2 signal pathway are still largely unknown. Presently, *MPK4/12* is down-regulated *HT1* in vitro which can phosphorylate *SLAC1* and shut down channel activities in response to elevated CO_2 concentration (Zhang et al., 2018). Although the critical components of the CO_2 stomatal movement signaling pathway have been identified (Zhang et al., 2018), many interactions, receptors, binding, and target remain unknown. A proposed CO_2 signaling pathway between MPK4/12, HT1, and CBC1/2 to S-type channel in response to CO_2 elevation has not been directly investigated. To further investigate the functions of these protein kinases, biochemical approaches such as protein interaction, kinase activation, and regulation are needed. In collaboration with another lab, the *E.coli* purified protein of MPK4 and HT1 are required for binding (Octet) and structural assays (cryo-EM). Octet analysis was designed to examine the binding of protein interaction in real-time. The experiment intends to test the binding of two proteins GST-MPK4 (~46 kDa) and His-HT1 (~50kDa) in real-time. The total of 1 mg of GST-MPK4 and 250 μg His-HT1 proteins were

produced. The purity of the protein was shown in Figure 10. The proteins produced will be used for further in vitro experiments to reveal protein functions. Further studies are needed to determine the detailed mechanisms of SLAC1 activation in presence of high CO_2 in plants.

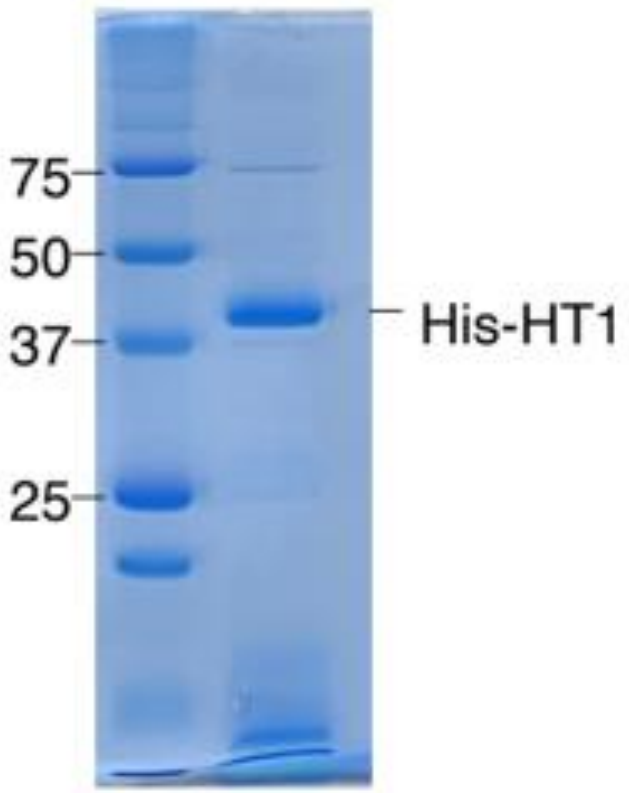

Figure 11. SDS Page result showing the purified of HT1 protein.

Recombinant His-HT1 proteins purified from *E.coli*.

Materials and Methods:

2.1 Protein Extraction from plants.

Seeds were sterilized using techniques described in chapter 1 and sowed on ½ MS medium containing plates. For DNA extraction, a buffer needed to be prepared which contains 0.5 mM of PMSF, 2 mM of DTT, and 500 μL of 100 mM MOPS-KOH (pH 7.5)/ 200 mM NaCl/ 5 mM EDTA/ 20 mM NaF for each DNA samples. Fifteen to twenty transgenic plants were grinding with a pestle and mortar on ice. The mixture was then transferred to 1.5 mL microcentrifuge tubes and centrifuge at 4^{o}C with 14000 RPM for 10 minutes. The supernatants were then transferred onto new microcentrifuge tubes on ice. By using spectrophotometry with the fixed wavelength of 595 nm, the OD of protein was determined. Using the Bradford Assay technique, and calculation formula (3.42 * OD = [protein] μg/μL), the protein concentration was determined.

2.2 Protein Extraction from E.coli.

This procedure required at least 3 days to perform. The plasmid vectors were generated by Dr. Yohei Takahashi and stored in -80^{o}C freezer after adding 80% glycerol.

Day 1: Preculture

- Streak the desired plasmid onto LB plates with vectors specific antibiotic and the culture will incubate to growth in the shaker at 200 rpm in 37 ^{o}C room.

Day 2: Scale-up

- Prepare freshly Liquid LB in Erlenmeyer flask with the desired volume.
- Add overnight preculture onto the flask with a vector-specific antibiotic. Incubate in the shaker at 200 rpm in 37 ^{o}C room.
- Measure the OD using a Spectrophotometer every 30 minutes until OD600 ~ 0.6.
- Add 1M IPTG stock solution to the flask so the final concentration is 0.5 mM.
- Incubate the cultures at 230 RPM in the 20 ^{o}C shaker overnight.

Day 3: Protein Purification.

- Divide the overnight *E.coli* cultures into 4 equal centrifuge bottles and immediately place them onto an ice bucket.
- Centrifuge the cultures at 6000 RPM for 10 minutes, discard the supernatant.
- Add the protein dilution buffer contains Hepes NaCl, DTT, and PMSF to each bottle.
- Gently dissolve the precipitate.
- Transfer solution onto 50 mL falcon tubes.
- Sonicate each tube with the amplitude of 18% with 30 seconds pulse on and 45 seconds pulse off.

- Immediately after sonication, add 20% Triton X-100 detergent stock solution was added onto tubes so the final concentration is 1% and incubate on ice for 30 minutes.
- The protein solutions were centrifuge at 12,000 rpm for 10 minutes at 4^{o}C.
- Transfer the supernatant to new falcon tubes and add 1% of glutathione sepharose (for GST tag) or Ni resin (for His tag) beads onto the solution.
- Incubate on the rotators at 4^{o}C for 2 hours.
- Centrifuge at 50xg for 3 minutes.
- Discard the supernatants.
- Add 12 mL Hepes NaCl Buffer and centrifuge at 50xg. Repeat washing 3 times.
- Add 1000 μL of Hepes NaCl Buffer to dissolve the pellets and transfer to 1.5 ml microcentrifuge tubes.
- Centrifuge at low speed for 45 seconds to separate the supernatant. Discard the supernatant.
- Add 80 μL of elution buffer.
    - HT1: Hepes His-Elution.
    - MPK4: Hepes GST-Elution.
- Dissolve the pellets and incubate for 10 minutes in the ice bucket.
- Centrifuge at low speed and transfer the supernatant to new microcentrifuge tubes.
- Add another 80 μL of elution buffer to the original microcentrifuge tubes, dissolve the pellets and incubate for 10 minutes in the ice bucket.
- Centrifuge at low speed and transfer the supernatant to the previous microcentrifuge tubes.

- Obtain new microcentrifuge tubes, transfer 130 μL of the supernatant to the tubes and add 80% glycerol to protein so the final concentration is 20%.

Measure protein concentration using Bradford Assay.

- Add 250 μL of Bradford solution to new microcentrifuge tubes.
- Add 5 μL of purified protein to the solution, mix well.
- Incubate the mixture for 5-10 minutes at room temperature.
- Measure the OD of protein using spectrophotometry at OD595.
- Protein concentration calculation:
    - [ ] = 3.42 x OD_{595} = μg/μL → convert to μL

2.3 SDS Page.

Prepare protein for SDS Page:

SDS Gel: smaller protein → use higher % gel.

A. 9 % Separation Gel - 5 mL (prepare in 13 mL tube)

Water	2.25 mL
4X separation buffer	1.25 mL
Acrylamide (30 %) (toxic when in liquid form)	1.5 mL
TE Med	5 µL
10 % Aps	50 µL

- Flip the tube several times to mix the solution well.
- Pour the solution in between 2 glass pieces.
- Using a micropipette, add about 1 mL of water on top slowly to avoid bubbles.
- Wait 10-15 minutes for the gel to solidify.

B. Stacking Gel - 1 mL

Water	605 µL
4X stacking buffer	250 µL
Acrylamide (30%) (toxic when in liquid form)	134 µL
TE Med	1 µL
10 % Aps	10 µL

- Discard the water on the gel first.
- Carefully add the solution on top of the separation gel.
- Slowly put the comb in all the way.
- Wait 10-15 minutes.

C. Transfer to SDS box

- Transfer the gel onto the SDS box, add ~450 μL of running buffer to it.
- Boil protein at 95 C for 3-4 minutes. Use 3x protein buffer (-20 C freezer), make 30 μL of solution.
- Load 2 μL of the marker.
- Run 20 mA for 60-90 minutes until the blue line runs to the bottom.

Blotting

- 6 filter papers (depend on the size of the gel) and 1 same size nitrocellulose membrane were prepared.
- Pour about ½ of transfer buffer to the square plate, in this order:
    - 3 filter paper → Gel → Nitrocellulose membrane → 3 filter paper.
- Transfer to the device and place them in this order:
    - 3 filter paper → Nitrocellulose membrane → Gel → 3 filter paper.
- Plugin and run the gel at 72 mA for 60-90 minutes.

Blocking

- Prepare 50 mL of T-TBS and 5% of Blotting Blocker (2.5 g).
- Incubate onto the rotator at room temp until everything is dissolved.

- After incubating for 1 hr, you can discard the filter paper and gel.
- Transfer the nitrocellulose membrane to the square plate, discard the solution then wash the membrane with distilled water 2-3 times.
- Pour about 30 mL of the solution made in step 1 onto the plate.
- Incubate on the shaker for 30 minutes.

1st Abs

- Discard the buffer.
- Wash the membrane 6-7 times with distilled water.
- Pour ~10-13 mL of specific tag solution onto the plate, incubate for 2 hours.
- Wash the membrane w/ T-TBS at least 3 times:
    - Pour T-TBS onto a well, transfer the membrane onto the well, incubate on the shaker for 5 minutes.
    - After 5 minutes, discard the T-TBS solution, pour the new T-TBS in and repeat.

2nd Abs

- Use 15 mL of Blocking solution + 3 μL of secondary antibody tag.
- Pour the solution onto the plate.
- Incubate on the shaker for 2 hours.
- Discard the solution, incubate on the shaker for 10 minutes with T-TBS solution, repeat this step 3 times.
- After washing, obtain the kit mix the 2 solutions together, the amount is based on the size of the membrane.

- Lay a saran wrap on the desk, place the membrane on it, swirl the mixture onto the membrane and incubate for 1 min.
- Obtain the clear sheet, place the membrane in the middle to detect.

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