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Overexpression of *Kalanchoë TIM23* Improves Heat and Drought Tolerance in *Arabidopsis thaliana* and Knockout of *TIM23* Reduces Growth in *Kalanchoë fedtschenkoi*

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To the Graduate Council:

I am submitting herewith a thesis written by Christopher H. Mendoza entitled "Overexpression of *Kalanchoë TIM23* Improves Heat and Drought Tolerance in *Arabidopsis thaliana* and Knockout of *TIM23* Reduces Growth in *Kalanchoë fedtschenkoi*." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology.

Hong Guo, Major Professor

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Overexpression of *Kalanchoë TIM23* Improves Heat and Drought Tolerance in *Arabidopsis thaliana* and Knockout of *TIM23* Reduces Growth in *Kalanchoë fedtschenkoi*

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ABSTRACT

Heat and drought stress are the leading causes of crop loss worldwide. The development of crops with elevated levels of heat and drought tolerance, which could sustain the production of food, feed, fiber, and biofuels on marginal lands, is therefore highly desirable. Heat stress usually causes protein dysfunction, and maintaining proteins in their functional conformations and preventing aggregation of non-native proteins are particularly important for plant survival under heat and drought stresses. Mitochondrial protein translocation and quality control pathways play an important role in refolding or removal of damaged proteins. Translocase of the inner mitochondria membrane 23 (TIM23) is responsible for importing a wide variety of mitochondrial proteins synthesized in the cytoplasm. However, the role of TIM23 in heat and drought stress tolerance has not been investigated in plants previously. In this study, we cloned two *TIM23* genes (*Kfe*TIM23-1 and *Kfe*TIM23-2) from *Kalanchoë fedtschenkoi*, which performs crassulacean acid metabolism (CAM) photosynthesis as an adaptation to water-limited environments. For gain-of-function analysis, two types of transgenic *Arabidopsis thaliana* plants were created by overexpressing *Kfe*TIM23-1 and *Kfe*TIM23-2, respectively, and these transgenic plants, along with wild-type *A. thaliana* plants, were then subject to drought stress and heat stress treatments. It was found that the

overexpression of *KfeTIM23-1* significantly enhanced heat and drought tolerance in *A. thaliana*. For loss-of-function analysis, one knockout mutant of *Kfe*TIM23-1 was created by using CRISPR/Cas9 technology. The DNA sequence of the *Kfe*TIM23-1 knockout mutant showed an insertion that disrupted the original amino acid sequence and consequently did not allow for the correct protein translation. As a result, a dwarfed phenotype was observed in the mutant line in comparison with the wild-type *K. fedtschenkoi* plants, suggesting that there may be a decrease in importing proteins into the mitochondria for the maintenance of CAM pathway. This research has a great potential for accelerating the genetic improvement of heat and drought tolerance in bioenergy and food crops.

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CHAPTER ONE: INTRODUCTION

Drought and heat stress are two major challenges faced by crop production world-wide. In the United States, Greenland Ranch, Death Valley, California holds a world record for intense heat on July 10, 1913, with the temperature reaching as high as 134°F (56.67°C). However, there is still life that flourishes in these extreme heat and drought conditions, and understanding how some organisms can survive in such environment is of considerable importance.

Plants such as *Kalanchoe fedschenkoi* and *Agave* can survive in hot and dry environments. They use Crassulacean Acid Metabolism (CAM) for photosynthesis. CAM is one of three photosynthetic pathways, which was evolved from C_3. CAM plants open their stomata during the night and fix CO_2 by using phosphoenolpyruvate carboxylase (PEPC). This leads to the formation of malic acid that is stored during the night in the vacuole (Cushman 2001). During the day, CAM plants close their stomata and convert the stored carbon source (malic acid) to CO_2, leading to an increase of CO_2 concentration around Rubisco (Cushman 2001). This process allows for CAM plants to have a water-use efficiency (WUE) that is much higher than C_3 and C_4 plants under the same conditions (Yang *et al.,* 2015). These evolutionary adaptations make CAM plants

the ideal model organisms to understand how they can tolerate the heat and drought environments using modern biological techniques.

Recently, Abraham *et al.* (2016) performed comparative analysis of gene expression between *Arabidopsis thaliana* (C_3) and *Agave* (CAM), and they found a reverse diel transcript expression pattern for some CAM-related genes during the day and night in *Agave* (CAM) relative to their orthologous genes in *Arabidopsis*. Since temperature is high during the day, it is possible that the ability of CAM plants to sustain drought and heat conditions may be related to the properties of the CAM genes that are activated during the day.

The mitochondria generate energy required for various biological processes in plants. In order for the mitochondrion to perform its function, it must import proteins from the cytoplasm. The proteins to be imported are unfolded, and their NH_2- terminal domains are recognized by the Translocases. The Translocase of the Outer Membrane (TOM) complex, which is composed of TOM20, TOM22 and TOM70, is considered to form the receptors that recognize the pre-sequence of proteins to be imported (Schmidt *et al.,* 2010). After the pre-sequences of the proteins are recognized by the receptors, the proteins go through the β-barrel channel which consists of TOM40. After traveling

through the outer membrane, the pre-sequences need to pass the inner membrane of mitochondrion through another complex called the Translocase of Inner Membrane 23 (TIM23) complex.

In order for the pre-sequence to travel through the TIM23 complex, it needs to have a NH_2 targeting sequence to be recognized by the N-terminus (hydrophilic domain) of TIM23. The TIM23 complex acts as a voltage-gated channel via TIM50 (Mokranjac *et al.,* 2005). TIM23 is the core of the TIM complex containing a hydrophobic region (C-terminus) which is embedded into the membrane. The TIM23 complex allows for the pre-sequence to go through the membrane by the help of TIM44, PAM16, PAM17, MGE1 and mtHSP70 (van der Laan *et al*., 2006). The proteins are then pulled down into the inner membrane by MGE1, and the NH_2 regions are removed by mitochondrial processing peptidase (MPP) (Gakh *et al.,* 2002). They then fold with the help of mtHSP70 with energy from ATP. (van der Laan *et al.,* 2006; Voos *et al.,* 2002; Schmidt *et al.,* 2010). In humans, it has been found that TIM23, 208 amino acids (aa) in length, consists of a hydrophilic N-terminus (1-74aa) and a hydrophobic C-terminus (75-208aa) (Zhang *et al.*, 2012).

For TIM23 in plants, including CAM plants, there is little information available. Unpublished data from Xiaohan Yang's lab at Oak Ridge National Lab showed that there is a difference in the diel transcript abundance of TIM23 in CAM and C_3 plants. Indeed, all the TIM23 genes of *Arabidopsis thaliana* (i.e., AT1G17530.1, AT1G72750.1, AT3G04800.1) are expressed during the night, while the two pineapple (CAM) TIM23 genes are both expressed during the day. It is of interest to note that for *Kalanchoë fedtschenkoi* Kaladp1244s0001.1 (*Kfe*TIM23-1) is expressed during the day, while Kaladp0037s0530.1 (*Kfe*TIM23-2) is expressed during the night. The exact reasons for these differences with the different plants are still not clear. Nevertheless, the expression of *Kfe*TIM23-1 during the day might lead to more stable TIM23 protein that is presumably required for the plant to sustain heat and drought conditions.

To the best of our knowledge, the connection of protein transport into the mitochondria with heat and drought stress tolerance of plants has not been examined previously. Here we hypothesize that *Kfe*TIM23-1 may make a contribution in heat and drought stress tolerance because it is expressed during the day when temperature is high. To test this hypothesis, the transgenic *A. thaliana* plants were created by overexpressing *Kfe*TIM23-1 and *Kfe*TIM23-2 individually (gain of function), and they were then subject to heat and drought stress treatments. It was found that overexpression of *Kfe*TIM23-1

significantly enhanced heat tolerance in *Arabidopsis thaliana*, while the effect of overexpression of *Kfe* TIM23-2 was not observed. Moreover, overexpression of *Kfe*TIM23-1 and *Kfe*TIM23-2 enhanced drought tolerance, and the effect of overexpression of *Kfe*TIM23-1 was more profound than that of overexpression of *Kfe*TIM23-2. Furthermore, a knockout mutant for *Kalanchoë fedtschenkoi* was generated for *Kfe*TIM23-1 using CRISPR/Cas-9 technology (loss of function) with an insertion in the coding sequence. It was found that the function of *Kalanchoë* TIM23-1 was lost, and the growth of *Kalanchoë* was reduced significantly. Collectively these results show the increase of heat and drought tolerance in the transgenic C_3 plants by the gain of function experiments and elucidate the importance of *Kfe*TIM23-1 in *Kalanchoë fedtschenkoi* for plant growth through the loss of function experiments.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Gene constructs for overexpressing *KfeTIM23-1* and *KfeTIM23-2.*

Kalanchoë TIM23-1 and *TIM23-2* coding sequences were obtained from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) and were synthesized as gBlocks fragments by Integrated DNA technologies (Coralville, Iowa, USA). The gBlocks fragments were inserted into binary vector pBI121 vector (Jefferson *et al.,* 1987), which contains 35S promoter to drive the overexpression of *KfeTIM23-1* or *KfeTIM23-2*. Specifically, the binary vector was digested with restriction enzyme XbaI and SacI (New England Biolabs Inc., Beverly, Massachusetts, USA). The digested fragments were separated by Agarose gel (1%) electrophoresis and the gel band corresponding to the vector backbone was collected and purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research Irvine, California, USA). The over-expression gene constructs p*KfeTIM23*-1-OE and p*KfeTIM23*-2-OE were constructed by combining of the purified binary vectorback bone with the *KfeTIM23-1* and *KfeTIM23-2* gBlock fragment, respectively, using the overlapping ends and using Gibson Assembly® Master Mix SacI (New England Biolabs Inc., Beverly, Massachusetts, USA). The assembled over-expression gene constructs were transformed *Escherichia coli* DH5α. Colony PCR was conducted with a pair of primers specific to the vector backbone. The primer sets used to

determine the transformation of p*KfeTIM23*-1-OE was 35 S Promoter with TIM23-1 R1 OE and TIM23-1 F OE with NOS-TER-R1. The primer sets used to determine transformation of p*KfeTIM23*-2-OE were 35 S Promoter with RP Tim23-2 and FP Tim23-2 OE with NOS-TER-R1 (Table 1). The PCR reaction conditions for amplification for *Kfe*TIM23-1 and *Kfe*TIM23-2 are 95℃ for 40 seconds, 58℃ for 40 seconds, 72℃ for 60 seconds, 30 cycles.

The *E. coli* cells containing plasmid p*KfeTIM23*-1-OE or p*KfeTIM23*-2-OE were grown over-night in LB media containing Kanamycin (50µg/mL). Plasmids were isolated from the over-night cell culture using Sigma Aldrich GenElute^{TM}Plasmid Miniprep kit (St. Louis, MO, USA). The purified plasmid p*KfeTIM23*-1-OE or p*Kfe*TIM23-2-OE was then transformed into *Agrobacterium tumefaciens* strain GV3101 by mixing the purified plasmids with the *Agrobacterium* competent cells followed by incubation at 37℃ for 5 minutes and 28℃ for 2 hours. Then the plasmid-*Agrobacteria* mixture was plated on LB Agar plates containing Kanamycin (50mg/L) and Rifamycin (100mg/L) and incubated at 28℃ for 48 hours. The transformed *Agrobacterium* cells were verified by colony PCR with a pair of primer sets used to determine the transformation of p*KfeTIM23*-1-OE was 35 S Promoter with TIM23-1 R1 OE and TIM23-1 F OE with NOS-TER-R1. The primer sets used to determine transformation of p*KfeTIM23*-2-OE were 35 S Promoter with RP

Tim23-2 and FP Tim23-2 OE with NOS-TER-R1 (Table 1). The PCR reaction conditions for amplification for *Kfe*TIM23-1 and *Kfe*TIM23-2 are 95℃ for 40 seconds, 58℃ for 40 seconds, 72℃ for 60 seconds, 30 cycles.

2.2 Plant transformation for overexpressing *KfeTIM23-1* and *KfeTIM23-2* in *Arabidopsis thaliana.*

Arabidopsis thaliana (Col-0) was transformed with the *Agrobacteria* cells containing p*Kfe*TIM23-1-OE, or p*Kfe*TIM23-2-OE using the floral dip method (Clough and Bent 1998). Transgenic plants with a ratio of 3 to 1 between Kanamycin resistance and the ones that were not resistant to Kanamycin were used in order to obtain homozygous lines for molecular characterization experiments.

Two transgenic plants were used in this study that contained *Kfe*TIM23-1 OE or *Kfe*TIM23-2. The morphology of the plants for *Kfe*TIM23-1 OE was bigger than the wild-type plant. Another important aspect was that it took longer for this plant to flower compared to the wild-type and transgenic *Kfe*TIM23-2 OE under normal conditions. As for *Kfe*TIM23-2 OE the morphology of the plant was similar to that of the wild-type, there was no size difference and there was no delay in flowering. For each *Kfe*TIM23-1

OE we used two mutant lines for this study. And for each of the lines we used triplicates for drought and heat stress treatments. For *Kfe*TIM23-2 we used two lines in this study that had the insertion of *Kfe*TIM23-2 OE. We used triplicates for *Kfe*TIM23-2 OE for drought and heat stress treatments.

2.3 Analysis of drought stress tolerance of *Arabidopsis* plants overexpressing *KfeTIM23-1* and *KfeTIM23-2.*

The *Arabidopsis* transgenic plants overexpressing *KfeTIM23-1* and *KfeTIM23-2,* along with the wild-type plants, were grown in CONVIRON Model BDW80 Plant Growth Room in pots containing commercial potting mix 3B (Sun Gro Horticulture, Vancouver, British Columbia, Canada) under a photon flux density of 100 µmol m^{-2} s^{-1} on a 14h light (23℃)/10h dark (23℃) cycle. The transgenic lines and control plants, in three biological replicates, were well watered followed by a 14-day drought treatment without watering. Images were taken with a digital camera daily around 8 am.

2.4 Analysis of heat stress tolerance in transgenic *Arabidopsis plants* overexpressing *Kfe*TIM23.

The *Arabidopsis* transgenic plants overexpressing *KfeTIM23-1* and *KfeTIM23-2,* along with the wild-type were grown in CONVIRON Model BDW80 Plant Growth

Room in pots containing soil mixture under a photon flux of 100 µmol m^{-2} s^{-1} on a 14h light (23℃)/10h dark (23℃) cycle, and then 5 week old plants were moved into Thermoscientific Precision incubator in pots containing soil mixture under a photon flux density of 100 µmol m^{-2} s^{-1} on a 14h light (42℃)/10h dark (42℃) for four days. Images were taken daily with a digital camera around 8 am.

2.5 Construction of CRISPR/Cas-9 Vector.

CRISPR/Cas-9 knockout of TIM23 guide RNAs were designed using software Protospacer^{WB} Workbench: CRISPR-CAS9 design (http://www.protospacer.com/). The guide RNA was selected by using the coding sequence of TIM23 from *Kalanchoë fedtschenkoi*. The guide RNA was selected to have no off-target site(s) and had a GC content of 50-60% excluding the PAM site. The guide RNA constructs were ordered from Integrated DNA technologies Coralville, Iowa, USA. The gRNA constructs were introduced into CRISPR/Cas-9 construct PKSE401 vector (Xing *et al.*, 2014). To synthesize the gRNA construct the following 3 primer sets were used: 1. TIM23_FP and TIM23_g1R 2. TIM23_g2F and TIM23_g2R 3. TIM23_g3F and TIMHindIII_LastR (Table 2). The construct was amplified with the following PCR conditions: 98℃ for 30 seconds, 56℃ for 30 seconds, 72℃ for 60 seconds, 32 cycles. Specifically, the binary vector was digested with HinDIII and rSAP restriction enzymes (New England Biolabs

Inc., Beverly, Massachusetts, USA). The digested fragments were separated by Agarose gel (1%) electrophoresis, and the gel band corresponding to the vector backbone was collected and purified using Zymoclean^{TM} Gel DNA Recovery Kit (Zymo Research Irvine CA, USA), and then Gibson Assembly was performed. *Agrobacteria tumefaciens* strain GV3101 containing the *Kfe*TIM23-1 CRISPR/Cas-9 plasmid was used for tissue culture transformation.

2.6 Agrobacteria-mediated transformation of Kalanchoë fedtschenkoi.

Transformation of *Kalanchoë fedtschenkoi* was performed according to the protocol developed by Cushman Laboratory at University of Nevada, Department of Biochemistry and Molecular Biology. The *Agrobacteria* containing the pKfeTIM23-1 CRISPR/Cas-9 vector was incubated for 12 hours in liquid LB containing Kanamycin (50 mg/L) and Rifamycin (100 mg/L). MS30 liquid media containing 0.2 mg/L indole acetic acid (IAA), and 100 µM thidiazuron (TDZ) was used to transform *Kalanchoë fedtschenkoi* by inoculation for 1 hour at room temperature. Leaves were transferred to co-cultivation media and placed in the dark for 4 days. After 4 days of incubation. the leaves were washed with MS Liquid media containing Timentin (100 mg/L) (Bioworld Dublin, Ohio USA), and transferred to MS30 Regeneration (1 mg/L thidizuron, 0.2 mg/L indole acetic acid, 100 mg/L Kanamycin). Plates were incubated at 16 hour light/ 8 hour

dark at 60-80 µmoles/m^2/light intensity at 20℃ which would formed the callus in 1 month (Figure 6A). Shoot formation was performed by transferring the callus to MS30 Shoot-induction media (1 mg/L benzylaminopurine, 0.2 mg/L indole acetic acid, 250 mg/L Timentin, 150 mg/L Kanamycin) and incubated at 16 hour light/ 8 hour dark at 60-80 µmoles/m^2/light intensity at 20℃ for 2-3 months for shoot formation (Figure 6B). Individual shoots were transferred into MS30 Rooting media (Sogo *et al.*, 2010) (150 mg/L Kanamycin, 250 mg/L Timentin) and grown under at 16 hour light/ 8 hour dark at 60-80 µmoles/m^2/light intensity at optimum temperature of 20℃ for a month for root formation (Figure 6C). After the formation of the root, the plants were transferred to soil (Figure 7).

2.7 Protein Sequence Alignments.

Amino sequence alignments for the different orthologs of *Kalanchoë* and *Arabidopsis* were performed using Clustal Omega online software using Muscle sequence alignment (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers *et al.*, 2011). Kaladp1244s0001.1 (*Kfe*TIM23-1), Kaladp0037s0530.1 (*Kfe*TIM23-2), AT1G17530.1, AT1G72750.1, AT3G04800.1 amino acid sequences were used.

2.8 Reconstruction of phylogenetic tree.

Phylogenetic tree was generated using Phylogeny fr. online software (http://www.phylogeny.fr/) Program PhylML 3.1/3.9 aLRT (Dereeper *et al.,* 2008; Dereeper *et al.*, 2010). Kaladp1244s0001.1 (*Kfe*TIM23-1), Kaladp0037s0530.1 (*Kfe*TIM23-2), AT1G17530.1, AT1G72750.1, AT3G04800.1 amino acid sequences were used.

2.9 Analysis of plant growth.

Plant measurements were conducted with Fiji Image J (Schindelin *et al.*, 2012).
A ruler with each plant was used as a reference for the measurements in centimeters. The plants were the horizontal span of the plants were measured by determining the diameter of the circle that would encompass the plants.

CHAPTER THREE: RESULTS

3.1 Comparison of TIM23 between Kalanchoë fedtschenkoi and Arabidopsis thaliana.

In *Kalanchoë fedtschenkoi* it was found that there are two isoforms of TIM23, while it is known that there are three isoforms for *Arabidopsis thaliana*. The alignments of the TIM23 sequences used Clustal Omega Alignment showed that there are more than 60% of amino acid residues that are conserved in the both *Kalanchoë* and *A.thaliana* TIM23 (Figure 1). For example, for Kaladp1244s0001.1 and Kaladp0037s0530.1, the percentage sequence identity is 62%, while for Kaladp1244s0001.1 and AT1G17530.1 the sequence identity is 64%.

The phylogenetic tree in Figure 3 shows that Kaladp0037s0530.1 (*Kfe*TIM23-2) is closely related to AT3G04800.1, while Kaladp1244s0001.1 (*Kfe*TIM23-1) is not. This may indicate that the role of the *Kfe*TIM23-1 and *Kfe*TIM23-2 orthologs might be different in *Kalanchoë fedtschenkoi*. Therefore, it would be of interest to introduce *Kfe*TIM23-1 and *Kfe*TIM23-2 into *Arabidopsis thaliana* and determine the change in heat and drought stress tolerance.

3.2 Overexpression of *Kfe*TIM23-1 in *Arabidopsis* enhances heat stress tolerance.

The plants of wild-type (Col-0), overexpression of *Kfe*TIM23-1 and overexpression of *Kfe*TIM23-2 are shown in Figure 4. Figure 4E shows that the transgenic plant with overexpression of *Kfe*TIM23-1 was still green after four days of exposure at constant 42℃, while the wild-type plant and the transgenic plant with overexpression of *Kfe*TIM23-2 turned to brown or yellow as shown Figure 4D and 4F respectively, under the same conditions. Together these observations show that the overexpression of *Kfe*TIM23-1 increases heat resistance. Additional experiments need to be performed for replications and statistical analysis in the future.

3.3 Overexpression of *Kfe*TIM23-1 in *Arabidopsis* enhances drought tolerance.

Figure 5 shows that overexpression of *Kfe*TIM23-1 and *Kfe*TIM23-2 in *Arabidopsis thaliana* leads to drought stress resistance in the plant at day 14. Specifically, the overexpression of *Kfe*TIM23-1 (Figure 5B) had significantly increased drought stress tolerance for the plant compared to the wild-type (Figure 5A). Moreover, the overexpression of *Kfe*TIM23-1 is superior in drought resistance compared to the overexpression of *Kfe*TIM23-2 (Figure 5C). Together these observations show that the overexpression of *Kfe*TIM23-1 increases drought tolerance. Additional experiments need to be performed for replications and statistical analysis in the future.

3.4 Loss-of-function of TIM23 in *Kalanchoë fedtschenkoi* reduces plant growth.

Knockout of Kaladp1244s0001.1 (*Kfe*TIM23-1) by CRISPR/Cas-9 during tissue culture of *Kalanchoë fedtschenkoi* by *Agrobacteria* transformation (Figure 7) resulted in a dwarfed phenotype having a measurement of 2.0 cm in the diameter of the plant compared to the wild-type with 7.5 cm in the diameter (Figure 7A). Both plants in the images had the same age of 2 months after tissue culture. The dwarfed transgenic *Kalanchoë* plant was sequenced in order to determine whether there was any mutations via CRISPR/Cas-9 (Figure 8). The corresponding sequences might contain mutations based on the guide RNA design (Figure 8A). The goal was to generate two mutations in each case. We did obtain mutations on both alleles of *Kfe*TIM23-1, and this suggests that the protein sequence for both alleles was disrupted. In the first allele, there was only one mutation observed as an insertion of a C in the coding sequence, leading to a frameshift mutation (Figure 8B). The frameshift mutation that is caused by a insertion in a coding sequence would result in a shift in the sequence, leading to a disruption of the original amino acid sequence. As a result of this mutation a there was no similarity with the original amino acid sequence (Figure 8C). In the second allele, there was an insertion that disrupted the amino acid sequence (Figure 8C). Collectively, the mutation would lead to a dysfunctional protein from *Kfe*TIM23-1 that may affect the protein import to the mitochondria and would therefore result in a dwarfed phenotype.

CHAPTER FOUR: DISCUSSION AND FUTURE EXPERIMENTS

All the three TIM23 genes of *Arabidopsis thaliana* are expressed during the night, while for *Kalanchoë fedtschenkoi Kfe*TIM23-1 is expressed during the day and *Kfe*TIM23-2 is expressed during the night. It would be of interest to examine whether the expression of *Kfe*TIM23-1 during the day when temperature is high might increase the heat and drought tolerant for the plant. Here we used TIM23 from *Kalanchoë fedtschenkoi* (*Kfe*TIM23-1 and *Kfe*TIM23-2) to understand the potential role of TIM23 from CAM for heat and drought stress. We generated the transgenic *A. thaliana* plants with overexpressing *Kfe*TIM23-1 and *Kfe*TIM23-2 individually (gain of function) and examined the change of heat and drought stress tolerance by comparing with wild-type *A. thaliana.* Moreover, we created the knockout mutant of *Kfe*TIM23-1 by CRISPR/Cas-9 in *Kalanchoë fedtschenkoi* and examined the effect on the plant growth. To the best of our knowledge, such experiments have not done previously.

Our results demonstrate that *Kalanchoë fedtschenkoi* (CAM) TIM23 have an advantage over the *Arabidopsis thaliana* (C_3) TIM23 for heat tolerance. Indeed, the overexpression of *Kfe*TIM23-1 was found to enhance the heat tolerance in transgenic *Arabidopsis thaliana* plants. Figure 5 shows that the overexpression of *Kfe*TIM23-1 and

*Kfe*TIM23-2 could lead to drought stress resistance as well compared to the wild-type plant, although the effect on drought tolerance was observed to be more prevalent for that containing *Kfe*TIM23-1 compared to one containing *Kfe*TIM23-2.

TIM23 is responsible for importing a wide variety of proteins synthesized in the cytoplasm into mitochondria. Our findings that the transgenic *A. thaliana* plants are more heat and drought tolerance might indicate that the *Kfe*TIM23-1 proteins may be still functional with the existence of heat and drought stress to import proteins into the inner membrane space of the mitochondria. By contrast, the *Arabidopsis thaliana* (C_3) TIM23 might be unable or less effective to do so under the same conditions. Interestingly, *KfeTIM23-1* is expressed during the day when temperature is high, but the exact reasons as to why *Kfe*TIM23-1 can lead to heat and drought tolerance are still not clear at the molecular level.

It should be pointed that, in addition to TIM23, there are other proteins in CAM plants which may also play a role for the maintenance of functional proteins for photosynthesis and for withstanding drought and heat stress during the day (Krause *et al.*, 2016; Berry *et al.*, 1980). For example, in C3 plans such as tomato, it has been observed that HSP40 (SlCDJ2) and HSP70 (cpHSP70) can protect Rubisco during heat stress.

Since CAM plants are expected to be under a constant exposure to heat and drought, HSP70 may play a role here as well. Interestingly, in the study by Yang *et al.* (2017) it was found that HSP70 was expressed during the day in the CAM plants (*Kalanchoë fedtschenkoi* [Kaladp0060s0296] and pineapple [Aco031458.1]), while in the C_3 plant (AT5G02490) it was only expressed during the night. Their results seem to suggest that, in addition to *Kfe*TIM23 studied here in this thesis, there may be a novel role of HSP70 for heat and drought stress tolerance in CAM plants as well. *Kfe*TIM23-1 activation during the day that we observed in this study might work together with the mitochondrial HSP70 for importing and refolding of proteins as well as removal of damaged proteins, which are important for plant survival under heat and drought stress.

The knockout of *Kfe*TIM23-1 showed a dwarfed phenotype compared to the wild-type (Figure 7). The sequence comparison (Figure 8) shows one line having an insertion in the coding sequence, indicating that the CRISPR/Cas-9 system worked in *Kalanchoë fedtschenkoi*. The existence of the dwarfed phenotype plant, which is much smaller compared to the wild-type suggests that its growth slowed down significantly without *Kfe*TIM23-1 in *Kalanchoë fedschenkoi*. The mutation was observed in the coding sequence in both alleles of *Kfe*TIM23-1, reflecting the guide RNA design where CRISPR/Cas-9 cleaved the coding sequence (Figure 8A). In the first allele (A1) (Figure

8B), there is an insertion of a C in the coding sequence, resulting in a frameshift mutation and leading to an amino acid sequence that has no similarity with the original sequence (Figure 8C). In the second allele (A2) there is an insertion resulting in a shift of the coding sequence which disrupted the original amino acid sequence (Figure 8C). The heat and drought stress tolerance of *Kfe*TIM23-1 knockout for *Kalanchoë fedschenkoi* is still unclear, and future experiments are still needed. Additionally, it is important to perform a complementation experiment to determine if we can recover the function of *Kfe*TIM23-1 and rescue the phenotype. Other experiments may include overexpressing all three *Arabidopsis thaliana* TIM23 in *A. thaliana* to determine whether they play a role in heat and drought stress.

It is important to determine whether the loss of *Kfe*TIM23-1 does affect heat and drought resistance for *Kalanchoë*. The future experiments may thus include heat stress tests in the loss of function mutants. Also, it is important to expose them at 42℃ and even higher temperatures like 55℃ and compare them to the wild-type *Kalanchoë.* If *Kfe*TIM23-1 is important for heat resistance, then we would see senescence on the leaves of the *Kfe*TIM23-1 knockout plants. There is a possibility that *Kfe*TIM23-2 might rescue the phenotype, and therefore the guide RNAs needs to be designed to knockout *Kfe*TIM23-2 as well. However, the knockout of both isoforms of *Kfe*TIM23 at the same

time could be lethal to the plant because at least one of them is likely needed to import proteins into the mitochondria.

In conclusion, the results on this study showed a novel role for *Kfe*TIM23 in heat stress and drought stress in transgenic *Arabidopsis thaliana.* TIM23 has never been shown before to have a role in heat and drought tolerance. This research has the potential in accelerating the genetic improvement of heat and drought tolerance in bioenergy food crops that could feed the overgrowing population.

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APPENDICES

APPENDIX A: TABLES

Table 1. Primers for colony PCR analysis of bacteria transformants used to overexpress *KfeTIM23-1* or *KfeTIM23-2* in *Arabidopsis thaliana.*

Primer Name	Sequence
35 S Promoter	5'-GACGCACAATCCCACTATCCTTCGC-3'
Tim23-1 F OE	5'-CGACAAAATTGCGTGTGAACCG-3'
Tim23-1 R1 OE	5'-ATTTTGTCGTATCGCCCGGCTCTGA-3'
FP Tim23-2 OE	5'-CTAGTGGACCCAGATCTGCC-3'
RP Tim23-2 OE	5'-CTGGGTCCACTAGCTGCTCGGTAA-3'
NOS-TER-R1	5'-GCCAAATGTTTGAACGATCGGGG-3'

Table 2. Primers for analysis of CRISPR/Cas-9 construct used to create the knockout mutant of *KfeTIM23-1* in *Kalanchoë fedtschenkoi.*

Primer Name	Sequence
TIM23_FP	5’-GTAAAACGACGGCCAGTGCCAAG CTTCGACTTGCCTTCCGCA-3’
TIM23_g1R	5’-ATTCGGCGATCGGTTCGCCACAAT CACTACTTCGACTCTAGC-3’
TIM23_g2F	5’-GTAGTGATTGTGGCGAACCGATCGCCGAAT GTTTTAGAGCTAGAAATAGCAAG-3’
TIM23_g2R	5’-AGGTAGCCGACTCCGGTGTACAATCT CTTAGTCGACTCTACCAAT-3’
TIM23_g3F	5’-GAGTCGACTAAGAGATTGTACACCGGAGT CGGCTACCTGTTTTAGAGCTA-3’
TIM23_HindIII _LastR	5’-AACCATGTTGACCTGCA GGCATGCAAGCT-3’

APPENDIX B: FIGURES

Figure 1. Protein sequence alignment for the TIM23 family members in *Kalanchoë* and *Arabidopsis.*

Conserved amino acid residues are designated by *. There are high sequence identities for these sequences. For instance, for Kaladp1244s0001.1 and Kaladp0037s0530.1 the percentage sequence identity is 62%, and for Kaladp1244s0001.1 and AT1G17530.1 the sequence identity is 64%. Sequence identity was determined by using Multiple Protein Sequence alignment NCBI. Multiple sequence alignment for Kaladp1244s0001.1 (*Kfe*TIM23-1), Kaladp0037s0530.1 (*Kfe*TIM23-2), AT1G17530.1, AT1G72750.1, AT3G04800.1 was performed using Clustal Omega online software (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Phylogeny of the TIM23 family members in *Kalanchoë* and *Arabidopsis*

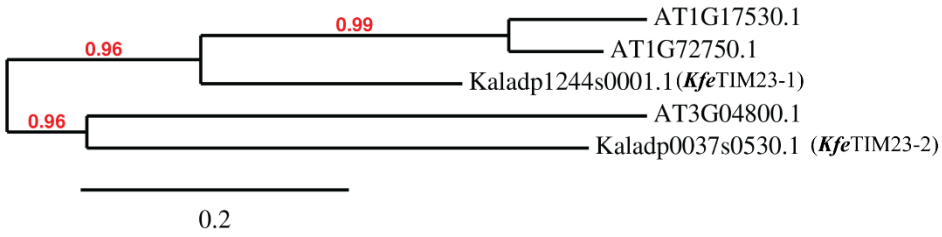

Figure 2. Phylogeny of the TIM23 family members in *Kalanchoë* and *Arabidopsis.*

Phylogenetic analysis for Kaladp1244s0001.1 (*Kfe*TIM23-1), Kaladp0037s0530.1 (*Kfe*TIM23-2), AT1G17530.1, AT1G72750.1, AT3G04800.1 using Phylogeny.fr online software (http://www.phylogeny.fr/index.cgi). The bar at the bottom of this tree (a value of 0.2) is the branch length in the amount of amino acid change that occur in the nodes at which deviations occur. The 0.2 value in the tree means that there is a 20% chance such as a substitution or mutation.

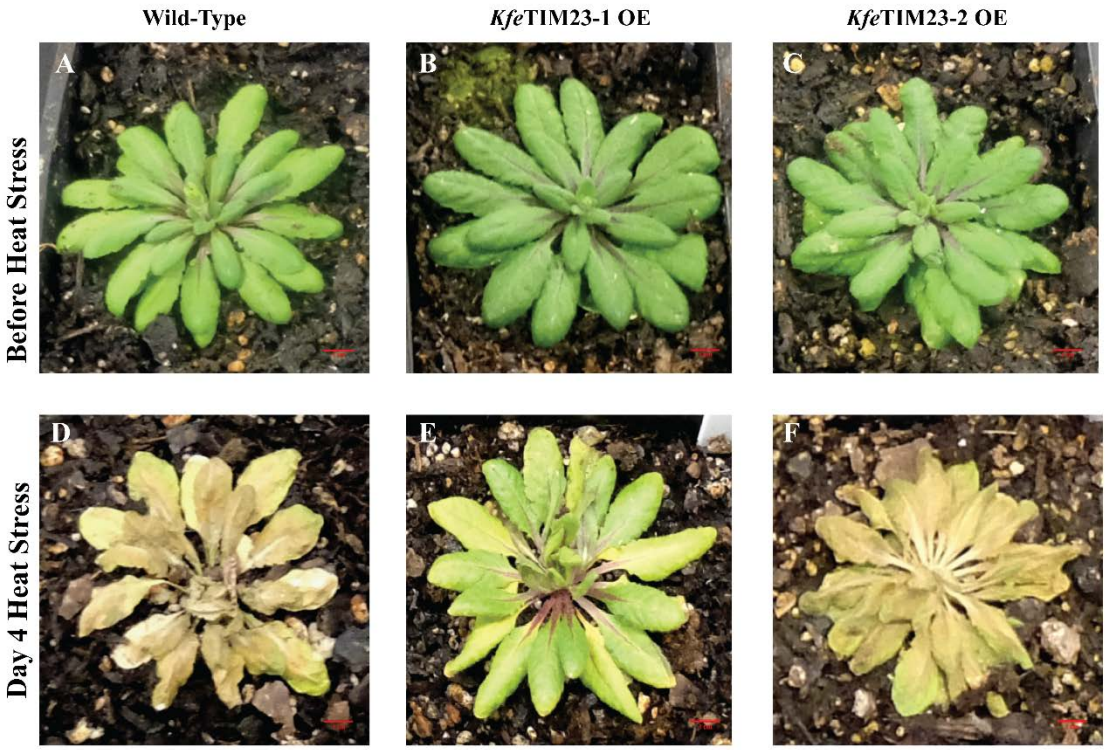

Figure 3. Effect of overexpressing *KfeTIM23* genes on heat stress tolerance in *Arabidopsis*.

(A) *Arabidopsis thaliana* wild-type before heat stress; (B) *Kfe*TIM23-1 overexpression in *Arabidopsis thaliana* before heat stress; (C) *Kfe*TIM23-2 overexpression in *Arabidopsis thaliana* before heat stress; (D) *Arabidopsis thaliana* wild-type exposed to 42°C for 4 days; (E) *Kfe*TIM23-1 overexpression in *Arabidopsis thaliana* exposed at 42°C for 4 days; (F) *Kfe*Tim23-2 overexpression in *Arabidopsis thaliana* exposed at 42°C for 4 days. Representative pictures from 3 replicates in each case.

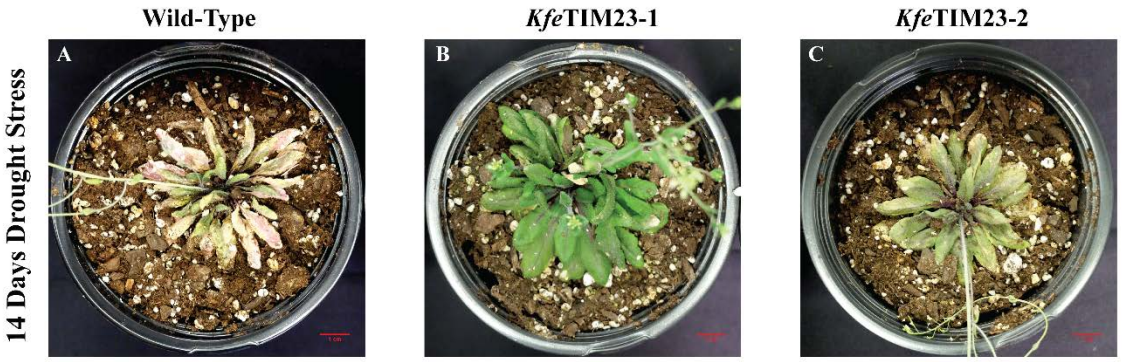

Figure 4. Effect of overexpressing *KfeTIM23* genes on drought tolerance in *Arabidopsis*.

(A) *Arabidopsis thaliana* wild-type with 14 days of drought stress; (B) *Kfe*TIM23-1 overexpression in *Arabidopsis thaliana* with 14 days of drought stress; (C) *Kfe*TIM23-2 overexpression in *Arabidopsis thaliana* with 14 days of drought stress. Representative pictures from 3 replicates in each case.

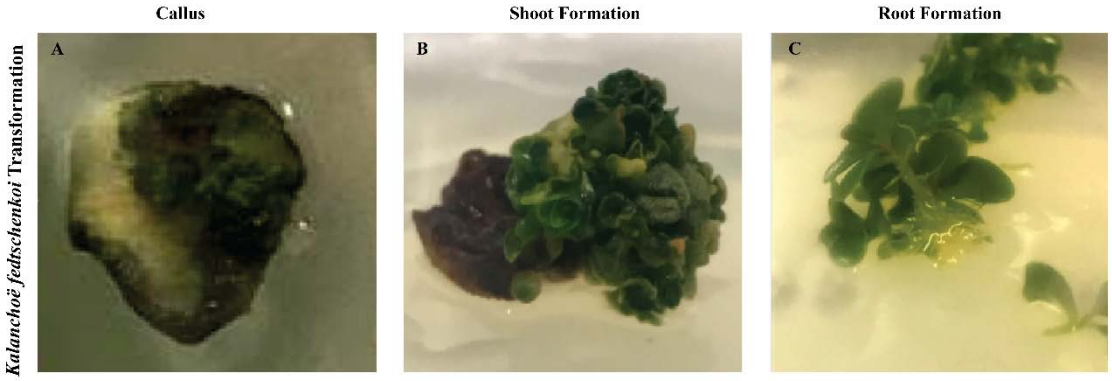

Figure 5. *Kalanchoë fedtschenkoi* transformation mediated by *Agrobacterium tumefaciens*.

(A) Callus formation of *Kalanchoë fedtschenkoi* after transformation with *Agrobacteria.* (B) Shoot formation after callus formation. (C) After shoot elongation for each individual shoot in root formation media.

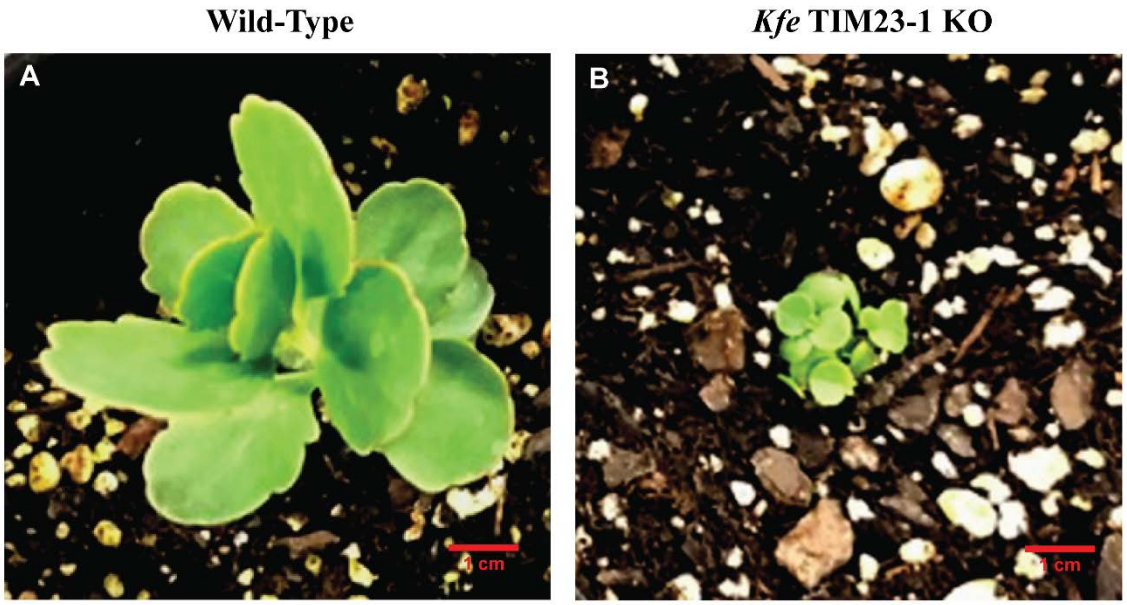

Figure 6. Growth phenotype of *TIM23-1* knockout mutant in comparison with wild-type *Kalanchoë fedtschenkoi* plant.

(A) Wild-type *Kalanchoë fedtschenkoi* plant with the measurement of 7.5 cm (B) *Kfe*TIM23-1 knockout by CRISPR/Cas-9 with the measurement of 2.0 cm. Both the wild-type and the knockout had the same age of 2 months after tissue culture.

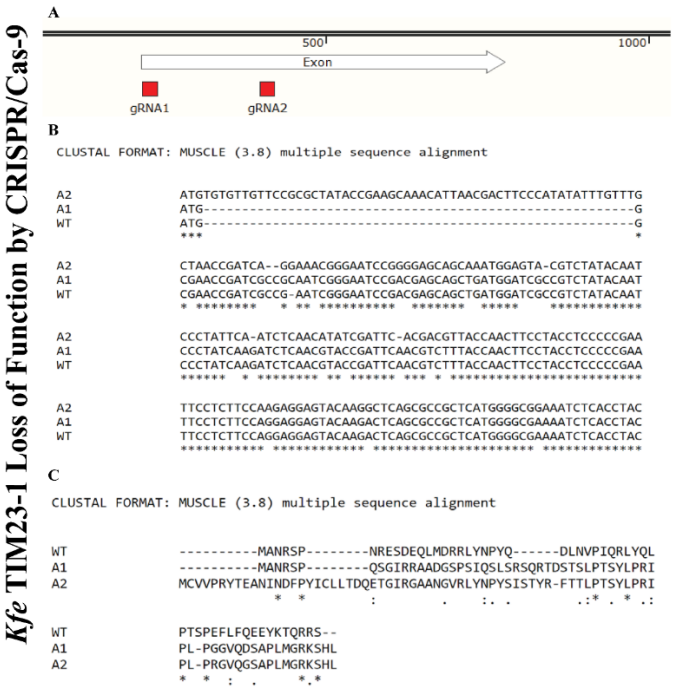

Figure 7. Sequencing confirmation of a *TIM23-1* knockout mutant of *Kalanchoë fedtschenkoi*.

(A) Guide RNA (gRNA) design on Kaladp1244s0001.1 coding sequence. (B) Nucleotide coding sequence of the knockout of *KfeTIM23-1* sequence shows an insertion of a C in the first allele (A1). In the second allele (A2) there is an insertion resulting in a frameshift. (C) The amino acid sequence was changed in allele 1 (A1) due to the insertion of a C in the nucleotide sequence. In the second allele (A2) the original amino acid sequence changed due to the insertion of the nucleotide sequence.

VITA

Christopher H. Mendoza was born in Mexico, he came to the United States as a kid. He is the first person in his family to go to college. He graduated from the University of Knoxville Tennessee with his Masters in Science. He enjoyed working at Oak Ridge National Laboratory for his studies.