THE EXPRESSION AND ANALYSIS OF A LYSINE-RICH WOUND-RESPONSE PROTEIN IN TOMATO PLANTS

by

Noah Kaplan

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Xing-Hai Zhang, Department of Biological Sciences, and has been approved by the members of his supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Masters of Science.

SUPERVISORY COMMITTEE:

Xing-Hai Zhang, Ph.D.
Thesis Advisor

David Binninger, Ph.D.

Mary Jane Saunders, Ph.D.

Rodney K. Murphey, Ph.D.
Chair, Department of Biological Sciences

Janet Blanks, Ph.D.
Interim Dean, Charles E. Schmidt College of Science

Deborah L. Floyd, Ed.D.
Dean, Graduate College

December 1, 2016
Date

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Most importantly, I would like to thank my friends and family whose support was vital in every way.

ABSTRACT

Author: Noah Kaplan

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Understanding the genetic regulation of the response to wounding and wound healing in fruiting plants is imperative to maintaining agricultural sustainability, preserving the quality of food supplies, and ensuring the economic viability of agriculture. Many genes are known to be induced by wounding, providing both structural repair and defense. The *KED* gene in tobacco (*Nicotiana tabacum*) has been shown to be induced by wounding. We have identified its homologue gene in tomato (*Solanum lycopersicum*) that we named *SlKED*. We have analyzed gene expression pattern of *SlKED* through tomato growth and development and in response to wounding as well as hormonal and inhibitor treatments. We found that the plant hormone ethylene played a major role in the expression of *SlKED*. To further identify evidence for physiological and transductional functions of *KED* and *SlKED*, the tobacco *KED* gene was introduced to tomato and overexpressed by the fruit tissue-active *PUN1* promoter from pepper (*Capsicum annuum*,). The expression of this gene was compared to the expression of the

native *SlKED* gene and other known wound response genes in both the wild-type and transgenic tomato plants. The upregulation of the native *SlKED* gene by wounding was significantly muted in the tobacco *KED*-expressing transgenic plants. The expression of other genes known to be associated with wound response transduction pathways was also altered. Our studies implicate the *KED* gene in defense mechanisms for mechanical stress in tomato plants.

DEDICATION

This manuscript is dedicated to the family that I lost while completing this degree. Grandma Shirley and Grandpa George, your support and unconditional love have made everything possible.

THE EXPRESSION AND ANALYSIS OF A LYSINE-RICH WOUND-RESPONSE PROTEIN IN TOMATO PLANTS

LIST OF TABLES ii

LIST OF FIGURES iii

INTRODUCTION 1

EXPERIMENTAL PROCEDURES 6

Plant material and growth conditions 6

Wounding and time-trial assay 6

Chemical treatment assay 7

Transcription analysis 7

Creation of transgenic plants 7

Verification of the transgene 9

RESULTS 11

Bioinformatics analysis 11

SlKED shows a low baseline expression in all tissue types 13

SlKED transcript levels peak 1 hour after wounding 13

SlKED expression is upregulated in the presence of ethylene analogues and downregulated by ethylene inhibitors 14

Generation and verification of transgenic plants 16

The overexpression of *KED* down regulates wound responsive genes 18

The fruit specificity of the *PUN1* promoter 20

DISCUSSION 21

REFERENCES 24

LIST OF TABLES

Table 1. Media used in the tomato transformation. 10

LIST OF FIGURES

Figure 1. The gene construct that was used in this experiment 9

Figure 2. The CLUSTAL Omega alignment of KED and SlKED. 11

Figure 3. An analysis of the normalized B-Factor in SlKED.. 12

Figure 4. The 3 dimensional structure of SlKED 12

Figure 5. The relative expression of *SlKED* by tissue type in plants without any mechanical stress 13

Figure 6. The relative expression of *SlKED* over 24 hours 14

Figure 7. The relative expression of *SlKED* under ethaphon treatment 15

Figure 8. Change in *SlKED* expression under AOA treatment in wild type plants after wounding 15

Figure 9. The expression of *SGT* under MeJA treatment in wild-type plants 16

Figure 10. The transgenic plants created for this study 17

Figure 11. Genotyping of the T0 transgenic plants..... 17

Figure 12. Genotyping of the T1 transgenic plants..... 18

Figure 13. Change in *SlKED* expression 1hr after wounding among wild type and transgenic plants..... 18

Figure 14. The relative expression of *ACO* and *SGT* in wild type and transgenic plants. 19

Figure 15. The relative expression of transgenic *KED* by tissue type. 20

INTRODUCTION

Plants have evolved sophisticated mechanisms for dealing with various environmental stresses, both biotic and abiotic, during their life cycle (Tian et al. 2014). Mechanical stress such as wounding by weather conditions, insects, and other animals is a constant threat that plants must deal with due to their sessile state (Savatin et al. 2014). *De novo* synthesis of plant hormones is known to occur upon wounding. Hormones such as jasmonates, abscisic acid (ABA), and ethylene initiate a set of interconnected signal transduction pathways that mediate the plant's response to wounding (Savatin et al. 2014). In particular, the gaseous hormone ethylene is involved in nearly every stage of plant growth and development (Light et al. 2016). Ethylene alone is a major component of leaf and flower senescence as well as root initiation (Klee 2002). However, many aspects of the molecular mechanisms of hormone-gene interactions remain unknown. Another hormone of note is jasmonic acid (JA). It is a hormone that is synthesized from fatty acid and plays an important role in the regulation of development in plants (Dhakarey 2016). It also plays a central role in a plant's response to various forms of stress, although JA itself is not an active signaling compound. It needs to be joined with an isoleucine for it to be recognized by its receptor, coronatine insensitive 1 (Dhakarey 2016).

We chose to investigate the involvement of *KED* expression with wound signaling pathways by treating wild type plants with several different known elicitors and

repressors of wound response. The wound response of tomato plants is a model system for the study of signaling pathways in higher plants (O'Donnell et al. 1996). With 25 years of previous studies, many genes have been identified as being directly induced by mechanical wounding (O'Donnell et al. 1996). Wound-responsive genes serve a number of functions that include resistance against insects, structural reinforcement of the cell wall, and hormone synthesis (Hara et al. 2000). We used methyl jasmonic acid (MeJA), a wound induced signal mediator (Niki et al. 1998); salicylic acid (SA), a wound induced signal mediator (Niki et al. 1998); acetyl salicylic acid (ASA), a jasmonate and ethylene inhibitor (O'Donnell et al. 1996); water (H_2O) as a control; sodium thiosulfate (STS) a competitive inhibitor to ethylene (Henstrand, et al. 1989); cobalt chloride ($CoCl_2$), an inhibitor of ethylene; aminooxyacetic acid (AOA), an ethylene inhibitor (Bradford et al. 1982); aminoisobutyric acid (AIB), an ethylene inhibitor (Bradford et al. 1982); and Ethephon, an ethylene-releasing compound when mixed with water (Granell et al. 1987).

Plants also employ the hypersensitive response in response to various types of stress (Johansson et al. 2015). The hypersensitive response is a complex process that involves a cascade of signals, programmed cell death, and the closing off of areas of tissue that cannot be repaired (Johansson et al. 2015). This is also a highly regulated process of gene expression. We chose to investigate the involvement of *KED* expression in the hypersensitive response by testing the expression of the gene that codes for scopoletin glucosyltransferase (*SGT*). Its transcripts have been shown to increase during the hypersensitive response in tobacco (Gachon et al. 2004).

To our knowledge, the only published study (Hara et al. 2000) identified *KED* as a wound responsive gene encoding a protein of 512 amino acids through a screening of

wound response transcripts in tobacco plants, and derived its name from the unusually rich content of lysine (K), glutamic acid (E) and aspartic acid (D), accounting for 34.7%, 25.0% and 12.5%, respectively, of its total amino acids. It has been suggested that *KED* played a role in general stress response. Its transcripts have been shown to accumulate rapidly, both locally and systemically, one hour after wounding in a transient but repeatable fashion (Hara et al. 2000). Transcripts return to undetectable levels after two hours. Hara et al. (2000) showed no change to the expression of *KED* under chemical treatment by known wound response antagonizers.

By searching the National Center for Biotechnology Information (NCBI) Genbank and the Solanum Genomics Network's DNA sequence database, we have identified a gene in tomato (*Solanum lycopersicum*) that is similar to the tobacco *KED* gene. It is a gene that has been predicted by the NCBI (LOC101265598) and is present in the SOL genomics project sequencing of the tomato genome (The Tomato Genome Consortium 2012). Our preliminary investigations showed that this tomato gene was indeed induced by wounding, therefore we tentatively name it *SlKED* with Sl referring to *Solanum lycopersicum*. Interestingly, with the notable exception of the family *Solanaceae,* we have not been able to find the *KED* homologue genes from any other plants whose genomes have been sequenced (such as *Arabidopsis, Oryza sativa*, or *Populus*), implying a possible unique evolutionary history for the *KED* genes.

Very little is known about the spatiotemporal regulation of the tobacco *KED* and the tomato *SlKED* gene or their physiological functions after wounding takes place. In this respect, we first characterized the *SlKED* gene expression patterns during a tomato life cycle and under different treatments of wounding and exposure to chemical elicitors

and inhibitors of wound responses. Then we generated transgenic plants overexpressing the tobacco *KED* gene. We investigated how the transgenic tomato plants responded to wounding in terms of expression of the endogenous genes, as compared to untransformed wild type plants.

In this study we chose to express the *KED* gene driven by the *PUN1* promoter. *PUN1* is a gene encoding an acetyltransferase that is believed to catalyze the final step in the synthesis of capsaicin in the pepper plant *Capsicum annuum* (Stewart et al. 2005, Ogawa et al. 2015). During the synthesis of capsaicin in pepper fruits, transcripts of *PUN1* rapidly accumulate in the placental tissue of pepper fruit (Keyhaninejad et al. 2013), suggesting that the *PUN1* promoter is strongly fruit tissue specific in pepper. Considering pepper and tomato both belong to the *Solanaceae* family, we sought to investigate the *PUN1* promoter's viability in use as a fruit specific promoter for gene expression in tomato.

To our knowledge, there has only been one published paper studying the *KED* gene (Hara et al. 2000). Our study constitutes the first time that a KED homolog gene, *SlKED*, has been identified and it is the first study to analyze the expression of either gene by using qPCR. This is also the first study to overexpress the *KED* gene in transgenic plants and compare the expression to endogenous wound responsive genes. This study will help us better understand how tomato plants respond to physical wounding and to further elucidate parts of the complex hormone mediated transduction pathways triggered by wounding.

This is also the first time that the *PUN1* promoter has been used to promote gene expression in tomato plants. This study will give us insight into the expression patterns of

the *PUN1* promoter and will add to the growing list of tools that scientists may use to study the effects of genes in a tissue specific manner. The fruit-specific promoter may also find utility for genetically improving nutrition and other qualities such as stress resistance in tomato fruits.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Tomato (*Solanum lycopersicum*, cv MicroTom) seeds were surfaced sterilized in 70% ethanol for 2 minutes and rinsed twice with deionized (DI) water. The seeds were then agitated in 10% hypochlorite (bleach) with 1 drop of Tween 20 for 20 min and rinsed with sterile DI water five times in a laminar flow hood. The seeds were then geminated in petri dishes on seed germination media (Table 1) and allowed to grow. After three weeks, the seedlings were transferred to seed germination media in Magenta (brand) growth boxes. After two weeks the plants were transferred to soil and grown in a temperature of 26°C, and a 16hr day and 8hr night cycle for one week. Six weeks after germination, samples were taken to study the expression of *SlKED* (Sun et al. 2006).

Wounding and time-trial assay

To determine if the accumulation of *SlKED* transcripts were induced by wounding, two wounding time trials were performed. Eight six-week old tomato plants were selected and their youngest fully extended leaf was wounded by pinching with 7-inch forceps. Samples were taken from the wounded leaf and an unwounded leaf of the same plant at 0, 0.5, 1, 2, 4, 8, 12, and 24 hr. Quantitative real time RT-PCR (qPCR) was performed on each sample and the results were normalized with the ΔΔCq calculation method (Bustin 2004) in which the reference genes were set to 100. Phosphoglycerate kinase (PGK) and elongation factor 1α were used as reference genes due to their

consistent expression in all parts of the tomato plant (Fletcher et al. 2004). A variety of other known wound responsive genes were also assayed using qPCR (Table 1).

Chemical treatment assay

Wounded and unwounded leaves were exposed to a series of chemical elicitors and inhibitors of plant wound responses (Table 2). Young leaves were clipped from 6-week old tomato plants and treated in eight different chemical treatments. Water was used as a control. The leaves were left to gently shake in the various treatments for one hour. After one hour, the samples were immediately dried and dropped in liquid nitrogen. qPCR was performed on each sample and the results were normalized with the ΔΔCq calculation method using PGK and elongation factor 1α as reference genes . This experiment was also carried out with two lines of transgenic plants (see below) (Hara et al. 2000).

Transcription analysis

To perform qPCR for detecting the transcription of the endogenous wound-responsive genes and the *PUN1-KED* transgene, total RNA was extracted from young leaves of the wild type and transgenic plants in similar growth conditions, using Qiagen Plant RNeasy mini kit. The RNA was converted into first-stranded cDNA using Maxima first strand cDNA synthesis kit (Thermos Scientific, USA) and random primers and oligo dT_{18}. Real-time qPCR using SYBR Green (Life Technologies, USA) was then carried out. Tomato PGK and elongation factor 1α served as references for qPCR (Bustin 2004).

Creation of transgenic plants

The *KED* gene and the *PUN1* promoter were cloned from tobacco and pepper (*Capsicum annuum*, cv. Serrano) plants respectively, and ligated together along with the

NPTII gene to confer resistance to kanamycin (Figure 1). This gene construct was fused into a PBI121 plasmid. The plasmid was then inserted into *Agrobacterium tumefaciens* strain EHA105 via electroporation.

We used a modified tomato transformation procedure adapted from Sun et al. (2006), Park et al (2003), and Chetty et al. (2012). The overnight-grown *Agrobacterium* was resuspended to an OD_{595} of 0.2 in liquid co-cultivation medium containing acetosyringone at 500 μM. Cotyledons taken from 8-11 day old wild type tomato seedlings (Scott et al. 1989) grown in aseptic conditions were incubated with the *Agrobacterium* culture for 10 minutes and were placed on a solid co-cultivation media described in Table 1. After 2 weeks of cultivation in the growth chamber, the cotyledons were placed on shoot induction media containing 100 mg/L kanamycin, as described in Table 1. The explants were left for 3 weeks and the media was changed if there was any sign of bacterial overgrowth. During this time the untransformed cells were killed by the kanamycin and the transformed cells grew into small shoots.

After the formation of shoots, the explants were removed and placed into shoot elongation media containing 100 mg/L kanamycin, described in Table 1. The explants were subcultured 2-3 times until the shoots were roughly 1.5-2 cm long. The shoots were then removed from the callus tissue and placed in the rooting media described in Table 1. The plantlets were allowed to fully form roots and an apical meristem before being moved into soil. The plants were grown in a temperature of 26°C, and a 16hr day and 8hr night cycle until they produced fruit.

As fruits ripened, seeds were harvested on a rolling basis and were cleaned by mixing in a 0.7% hydrochloric acid solution for 45 minutes, rinsed thoroughly in water

and dried for 24 hours. The seeds were then surface sterilized as described above and germinated on seed germination media (Table 1) containing 100 mg/L kanamycin. The resistant plants were selected and allowed to grow for 2 weeks in Magenta boxes containing germination media (Table 1). The plants were then transferred to soil and grown in a 16hr day and 8hr night cycle.

Verification of the transgene

The transgene in the T0 generation was verified by genotyping using qPCR. A 50 mg leaf sample was taken from each line of transgenic plants. Genomic DNA was extracted from the sample using Qiagen Plant DNeasy mini kit and qPCR was performed on each sample as described above. The plants that were positive in genotyping, suggesting the presence of the tobacco *KED* gene, were grown to maturity. T1 seeds were harvested and the seedlings were genotyped again via qPCR to verify the presence of the *KED* transgene. These T1 plants were likely homozygous and were used for further studies of wounding response (Bustin 2004).

Figure 1. The gene construct used in this experiment. This gene construct has *KED* being driven by the *PUN1* promoter from a pepper.

Table 1. Media used in the tomato transformation.

Seed Germination Media	
1 x MS Salts + Gamborg Vitamins	
Sucrose	30 g/L
Phytagel	2.7 g/L
pH	5.8
Co-Cultivation/ Pre-Cultivation Media	
1 x MS Salts + Nitsch Vitamins	
Trans-Zeatin Hydrochloride	1 mg/L
Sucrose	30 g/L
Agar	8 g/L
6-Benzylaminopurine (6Ba)	1 mg/L
Acetosyringone	500 uM
pH	5.8
Callus Induction/ Selection Media	
1 x MS Salts + Nitsch Vitamins	
Trans-Zeatin Hydrochloride	2 mg/L
Sucrose	30 g/L
Agar	8 g/L
IAA	0.5 uM
Folic acid	0.05 mg/L
Myo-Inositol	100 mg/L
Kanamycin	100 mg/L
Timentin	400 mg/L
Carbenicillin	500 mg/L
pH	5.8
Shoot Elongation Media	
1 x MS Salts + Nitsch Vitamins	
Trans-Zeatin Hydrochloride	1 mg/L
Sucrose	30 g/L
Agar	8 g/L
IAA	0.5 uM
Folic acid	0.05 mg/L
Myo-Inositol	100 mg/L
Kanamycin	100 mg/L
Timentin	400 mg/L
Carbenicillin	500 mg/L
pH	5.8
Rooting Media	
1 x MS Salts + Gamborg Vitamins	
Sucrose	30 g/L
Phytagel	2.7 g/L
IAA	0.5 uM
pH	5.8

RESULTS

Bioinformatics Analysis

The 512 amino acid sequence of tobacco KED was aligned with the 427 amino acid tomato SlKED sequence using Clustal Omega (Sievers et al. 2011). The results of the alignment are shown as Figure 2. The results indicate 65.4% identity conservation of identical and similar residues between the two gene sequences.

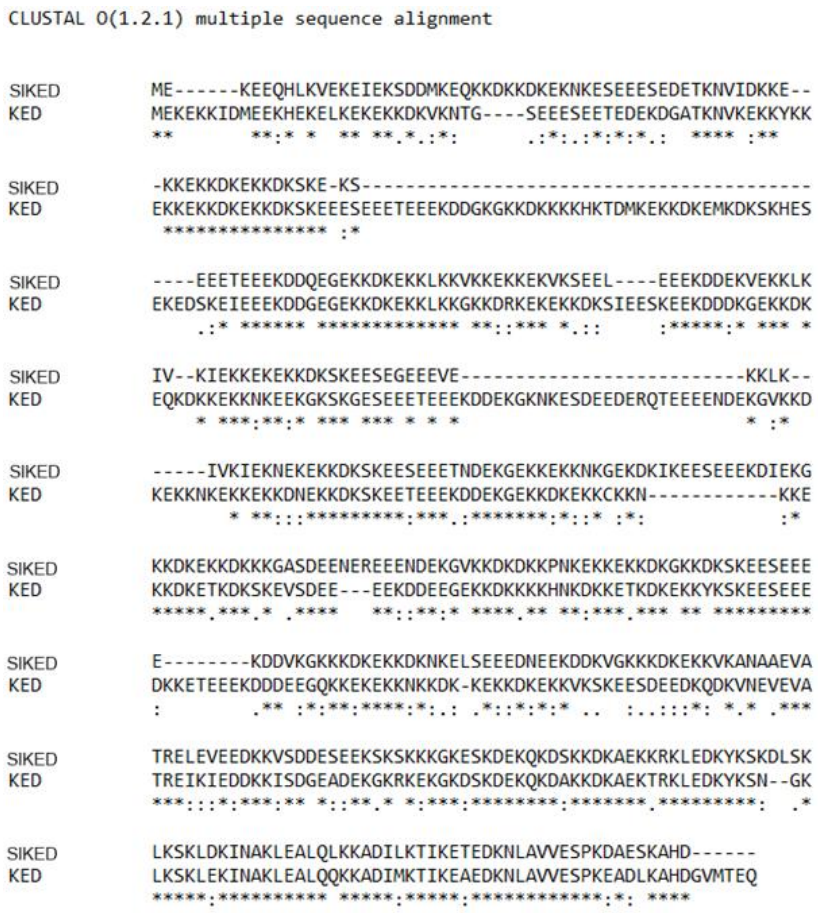

Figure 2. The CLUSTAL Omega alignment of KED and SlKED * indicates a fully conserved residue. **:** indicates the conservation of strongly similar groups. **.** indicates conservation of weakly similar groups. Blank spaces indicate no consensus.

Analysis of the SlKED protein structure was performed by using I-Tasser from the University of Michigan (Roy et al. 2010). The results indicate that the tertiary structure of the protein is very unstable. Figure 3 shows that the B-factor, which assess the thermal mobility of amino acid residues, is high indicating an unstable tertiary structure. Several possible 3D models of the tertiary structure were produced. The structure that showed the highest degree of confidence is shown in Figure 4. In a blast of the *KED* and *SlKED* sequences, we were unable to locate any similar genes in any fully sequenced species other than *Solanaceae* species.

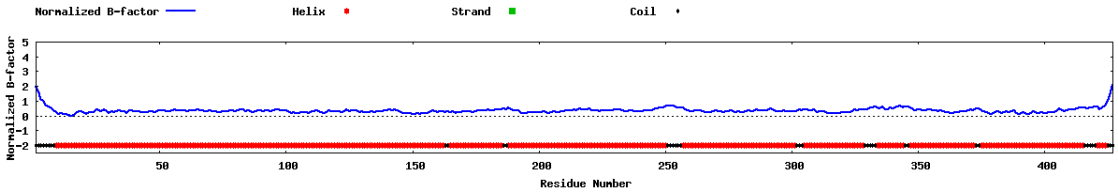

Figure 3. An analysis of the normalized B-Factor in SlKED. The lack of negative values indicates very low stability of the tertiary structure.

Figure 4. The 3 dimensional structure of SlKED. A. The 3D structure with the N-terminal colored blue and the C-terminal colored red.

SlKED shows a low baseline expression in all tissue types

Samples of the leaf, stem, fruit, and root were taken from an unwounded plant to establish the baseline tissue specificity of *SlKED* expression. While the baseline expression of *SlKED* was considerably lower than after a wounding event, results indicate that expression of *SlKED* in the root and the fruit were considerably higher, but significant expression was found in the leaf and stem as well. This can be seen in Figure 5.

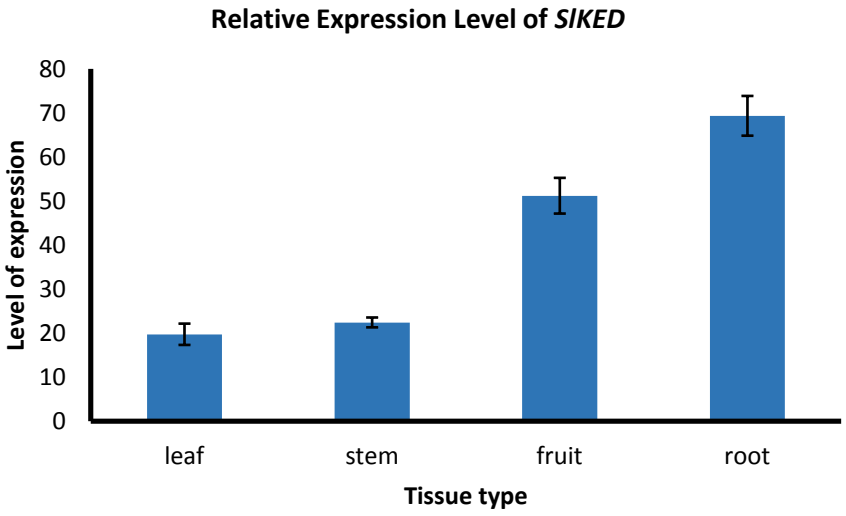

Figure 5. The relative expression of *SlKED* by tissue type in plants without any mechanical stress. The root shows the highest level of expression followed by fruit. Stem and leaf show significantly lower expression

SlKED transcript levels peak one hour after wounding

Determining the timing of peak *SlKED* expression was carried out in a time course assay. We found that *SlKED* expression peaks one hour after wounding and quickly dissipates to baseline levels by two hours (Figure 6). On average, we found a

115-fold increase (with some variation between plants) in the expression of *SlKED* one hour after wounding.

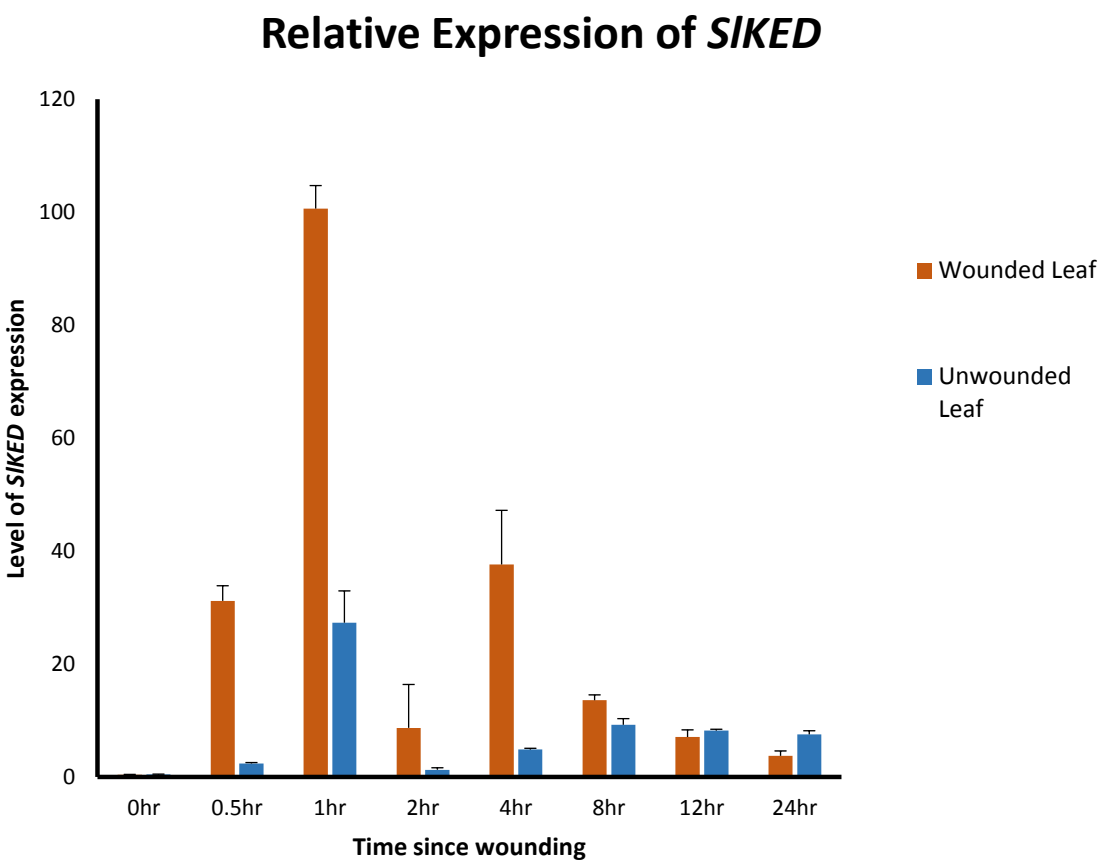

Figure 6. The relative expression of *SlKED* over 24 hours. *SlKED* expression increases at 0.5 hours and peaks at one hour. By two hours expression of *SlKED* has returned to baseline levels.

SlKED expression is upregulated in the presence of ethylene analogues and downregulated by ethylene inhibitors

Wounded and unwounded leaves of the wild type plants were exposed to chemical elicitors and inhibitors of plant wound responses of varying types. The ethylene-releasing ethephon was used to treat unwounded leaves from six-week-old plants. Our results indicate that expression of *SlKED* was increased by four fold following exposure to ethephon, (Figure 7). When treated with aminooxyacetic acid

(AOA), a competitive inhibitor to ethylene, transcription of *SlKED* was inhibited (Figure 8).

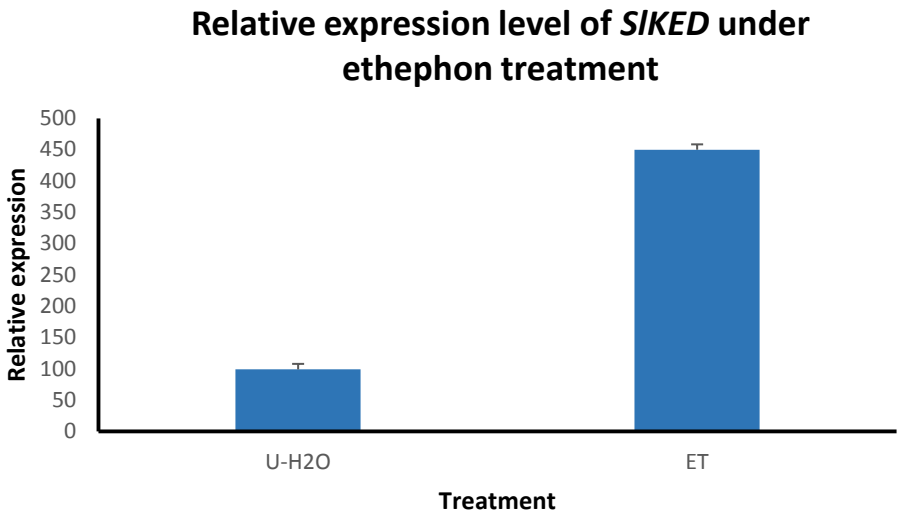

Figure 7. The relative expression of *SlKED* under ethephon treatment. *SlKED* shows an increased expression when exposed to ethephon (ET). H_2O was used as a control. U: unwounded

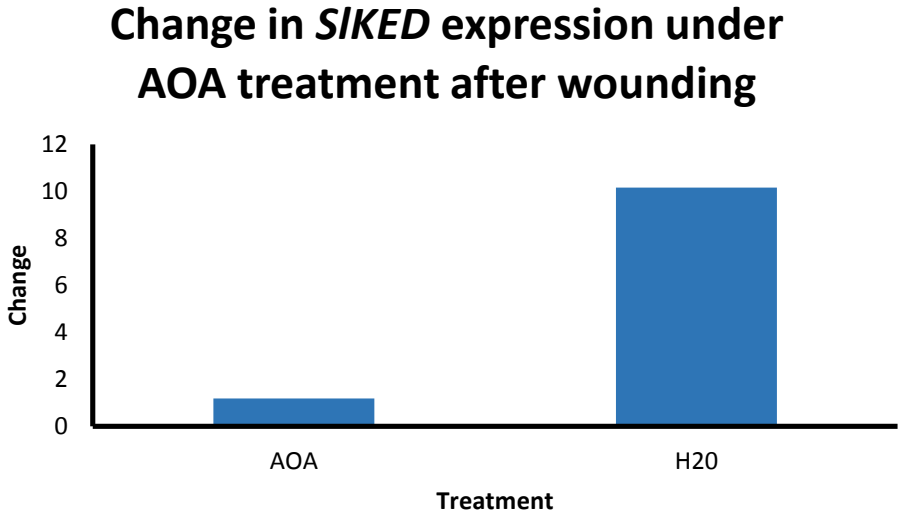

Figure 8. Change in *SlKED* expression under AOA treatment in wild type plants after wounding. *SlKED* expression was inhibited under AOA treatment in wild type plants. Water was used as a control treatment.

SGT expression is upregulated by methyl jasmonic acid

Scopoletin glucosyltransferase (*SGT*) has been shown to be upregulated during the hypersensitive response in tobacco plants (Gachon et al. 2004). We found that *SGT* is also upregulated in the presence of methyl jasmonic acid (Figure 9).

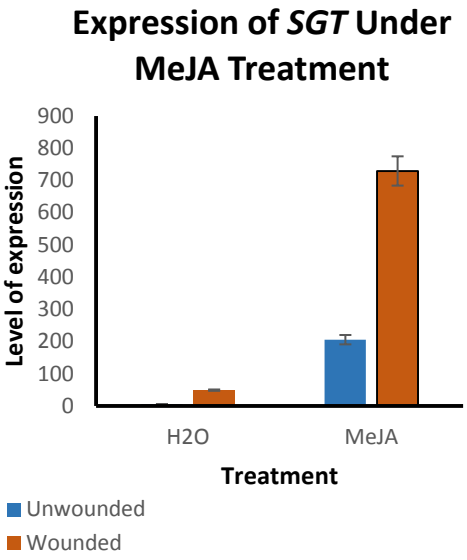

Figure 9. The expression of *SGT* under MeJA treatment in wild-type plants. The expression of *SGT* was highly upregulated after exposure to MeJA

Generation and verification of transgenic plants

Ten independent transgenic lines overexpressing the *KED* gene were created via *Agrobacterium* transformation and the lines used in this study are shown in Figure 10. Initial shoots were selected on media containing kanamycin and all kanamycin resistant plants were allowed to grow to maturity. Upon fruiting all the plants reproduced normally and produced seeds. Transgenic lines in the T0 generation were verified via qPCR. Figure 11 shows the gene dosage level of nine T0 lines of transgenic plants. Line A is not shown as it showed an unusually high gene dosage that is assumed to be caused by remnant *Agrobacterium* cells still present in the tissue.

At the time of writing, two lines of plants from the T1 generation have been verified. The T1 seeds were germinated on germination media containing kanamycin and the surviving plants were verified via qPCR. Figure 12 shows that line A – 1, line 20 – 1, and line 20 – 2 are likely homozygous, having roughly double the gene copy level as line A-2 which is likely heterozygous.

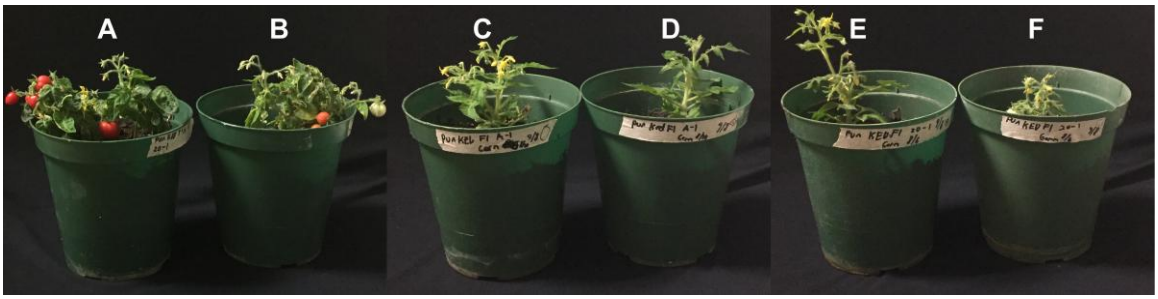

Figure 10. The transgenic plants created for this study. A: The T0 generation of line 20. B: The T0 generation of line A. C-D: The T1 generation of line A. E-F: The T1 generation of line 20.

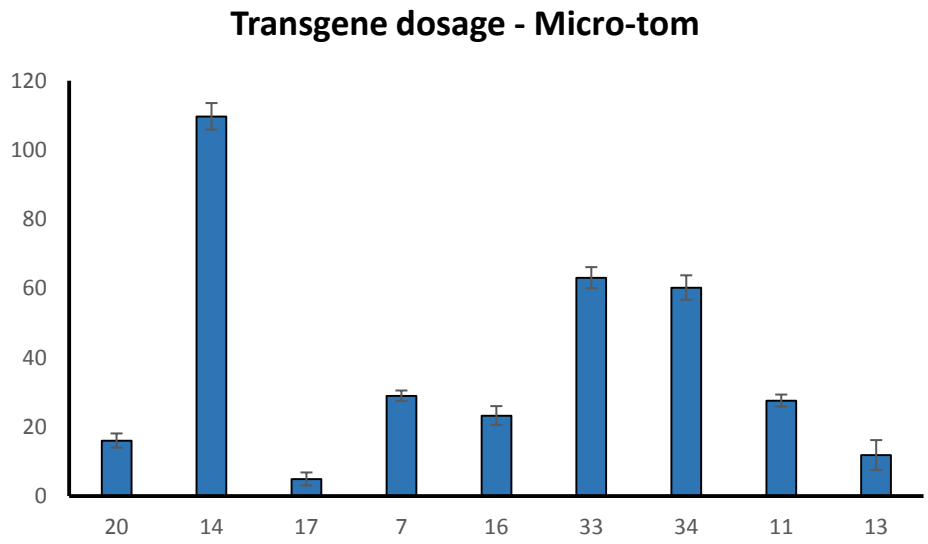

Figure 11. Genotyping of the T0 transgenic plants. 10 positive lines of transgenic plants were verified at the T0 level.

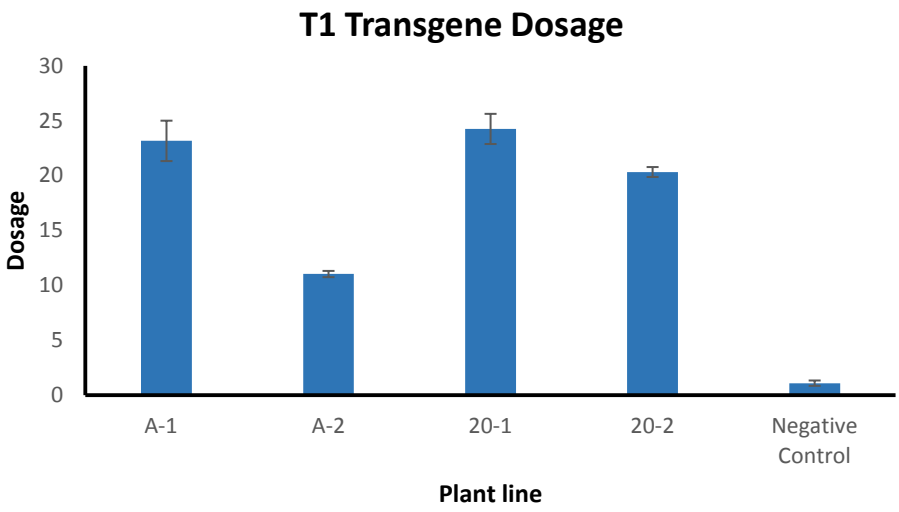

Figure 12. Genotyping of the T1 transgenic plants. Two plants from two lines were verified via qPCR. Line A – 1, line 20 – 1, and line 20 – 2 are likely homozygous. Line A-2 which is likely heterozygous.

The overexpression of KED down regulates wound responsive genes

The overexpression of the *KED* gene in the transgenic plants has a down regulating effect on several wound responsive genes. The native *SlKED* gene showed a significantly smaller increase in expression after wounding in the presence of the transgene. The wild type plant showed a 115.6 fold increase, while line 20 and line A showed a 35.5 and 34.2 fold increase respectively. This is demonstrated in Figure 13.

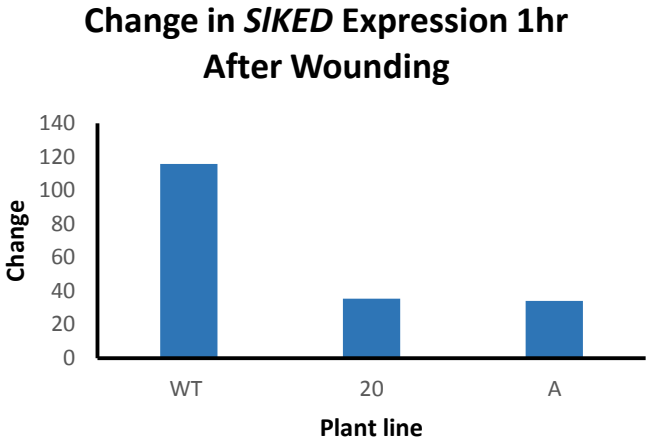

Figure 13. Change in *SlKED* expression 1hr after wounding among wild type and transgenic plants. The wild type plant shows a 115.6 fold increase while line 20 and line a show a 35.5 and 34.2 fold increase respectively.

The wound response genes 1-aminocyclopropane-1-carboxylate oxidase (*ACO)* and *SGT* are also down regulated in the *KED* expressing transgenic plants. Figure 14 shows a decrease of expression in *ACO* in both unwounded and wounded samples as compared the wild type plants. *SGT* shows the similar expression as wild-type plants in unwounded samples but its transcripts failed to accumulate upon wounding in the transgenic plants.

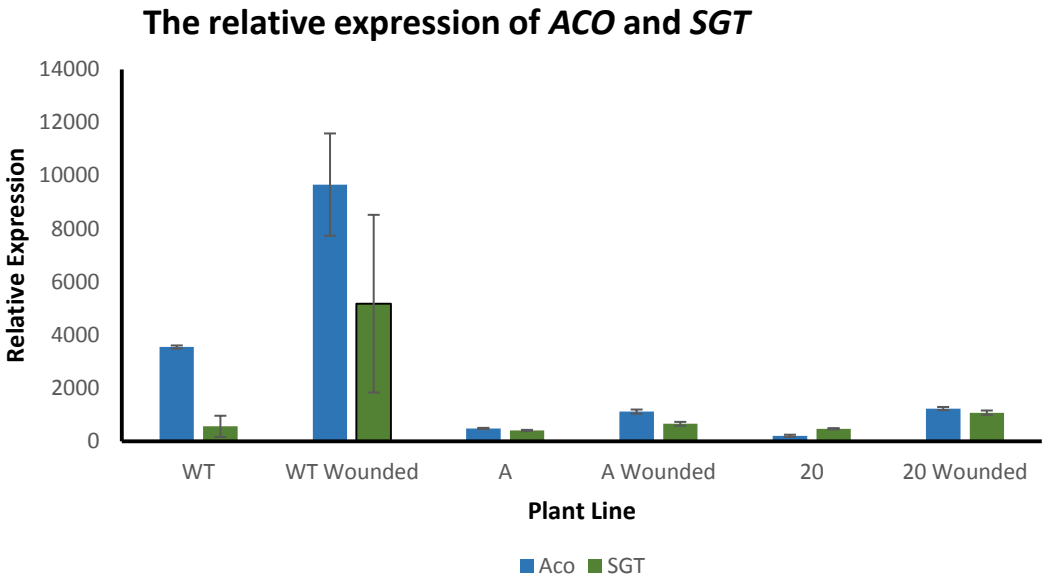

Figure 14. The relative expression of *ACO* and *SGT* in wild type and transgenic plants. The transgenic plants show suppressed expression of *ACO* in both wounded and unwounded samples and a suppression of *SGT* in wounded samples.

We showed that the *SlKED* gene is upregulated in the presence of ethylene (Figure 7). *ACO* is an ethylene precursor gene (O'Donnell et al. 1996). The lower accumulation of *ACO* transcripts correlates with a lower rate of ethylene synthesis. *SGT* is involved in the hypersensitive response (Gachon et al. 2004). The lower accumulation of *SGT* transcripts upon wounding may reflect a lesser hypersensitive response and cell death. Therefore, in our transgenic plants, the presence of *KED* may ameliorate wounding

stress response, resulting in lower accumulation of *SlKED*, *ACO* and *SGT* transcripts than observed for the wild type plants.

The fruit specificity of the PUN1 promoter

We performed qPCR on samples of the leaf, flower, ripened fruit, and immature fruit of a *KED* expressing transgenic plant driven by the *PUN1* promoter (Stewart et al. 2005). We found that the *PUN1* promoter was able to provide fruits specificity of up to 294-fold higher in the fruit as opposed to the flower and the leaf. These results are shown in Figure 15.

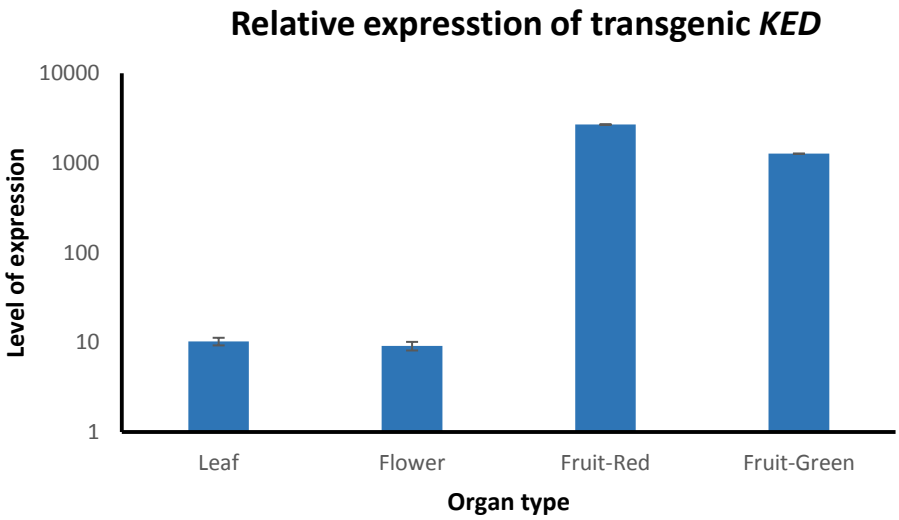

Figure 15. The relative expression of transgenic *KED* by tissue type. Expression in the fruit was increased as much as 294-fold compared to the flower and leaf.

DISCUSSION

Our computational analysis of the structure of *SlKED* through basic bioinformatics analysis indicates that the *SlKED* protein has unstable tertiary structure. This is consistent with the assumptions of Hara et al. (2000) that the tobacco *KED* has an unstable tertiary structure based on the lack of hydrophobic amino acid clusters necessary for protein folding. While we can use this as evidence of *KED* and *SlKED* having similar functions, we are unable to surmise any gene function from our bioinformatics research.

Our findings on the baseline expression of *SlKED* in tomatoes were consistent with the *KED* study in tobacco (Hara et al. 2000). We found that the timing of *SlKED* transcripts accumulation of one hour after wounding was the same as the *KED* gene in tobacco, providing further indication that these genes are homologs of each other. The lack of similar sequences found in other sequenced plants indicates an evolutionary pathway shared between tobacco and tomato originating in a common ancestor (Giacopelli 2015). We did find transcripts in the unwounded leaves, roots, flowers, and fruits of tomato in a pattern that differed in tobacco. In their study, Hara et al (2000) were unable to identify any transcripts of *KED* in unwounded plants. This is probably due to the difference in methodology, and the facts that qPCR is far more sensitive than the Northern blotting used by Hara et al. (2000).

We showed that the expression of *SlKED* can be induced solely by ethylene without wounding, which has not been shown in the previous tobacco study and implicates ethylene and *SlKED* in being part of the larger wound response transduction pathways.

Gene expression patterns of the transgenic plants are dramatically different than those of wild type plants. In tomato expressing the tobacco *KED*, the native *SlKED* gene, as well as two other key wound responsive genes *ACO* and *SGT*, showed a muted expression upon wounding when compared to the untransformed plants, suggesting that the wound response of transgenic plants is weaker than that of the untransformed plants. One explanation for this is that the *KED*-expressing plants may not be perceiving the wounding as stressful as the wild type plants, implicating a role of *SlKED* in protecting the cells against wounding. A less elevated level of transcription of the endogenous wounding responsive genes such as *SlKED*, *ACO* and *SGT* (Figures 13 and 14) may indicate a status of wound tolerance for these transgenic plants, since the wild type plants exhibited an acute, rapid, and large scaled increase in the transcription of these genes in response to wounding. We also believe that *KED* and *SlKED* may play a role in the hypersensitive response due to its interaction with *SGT*, a gene that is associated with the hypersensitive response (Johansson et al. 2015). *SGT* is also upregulated by methyl jasminate and thus, *KED* and *SlKED* may be mediated in part by jasmonates (Figure 9) as well as ethylene.

Our study revealed that the *PUN1* promoter drove *KED* transcription in all the plant organs tested but the expression was considerably higher in the fruit than in the flowers or leaves. This study constitutes the first time that the *PUN1* promoter has been

used for gene expression in tomato plants and the *PUN1* promoter has shown to be a suitable method of driving fruit specific expression in tomato plants while also showing a degree of expression throughout the plant.

In this study we have shown that the tobacco *KED* and tomato *SlKED* are likely homologous. They are expressed in the same pattern after wounding and have a 65.4% identity conservation. The overexpression of *KED* in tomato causes a diminished wound response indicating that these genes play a major role in the response to wounding. Further studies to determine the function of the gene and the benefits of overexpressing it may include post wounding cell death assays, membrane stability assays, and insect feeding assays. We are planning to examine whether there is a difference in cell death response (Baker et al. 1994) caused by wounding between the wild type and the transgenic plants. The high lysine content of *SlKED* protein may also have utility in improving the general nutrition of the plant and the fruit specificity of the *PUN1* promoter may be an important tool in fruit focused studies.

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