

Analysis of pollen-pistil interactions to model reproductive thermotolerance in tomato

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Abstract

Under current global circumstances, developing heat-tolerant crops has become of pinnacle importance. *S. lycopersicum*, the commonly cultivated tomato variety, is one of the most valuable vegetable crops and a widely used plant research model organism. Successful pollination in tomato under heat stress requires thermotolerant male pollen tube to carry the sperm cells and grow within the female pistils to complete fertilization and form seeds. Accordingly, tomato accessions with shorter pistils were hypothesized to have greater thermotolerance because the pollen tubes travel a shorter distance and have fewer opportunities for premature bursting events, both aspects likely to be experienced by pollen tubes grown under heat stress. In addition, shorter pollen tubes were hypothesized increase thermotolerance by reducing pollen tube travel distance, decreasing the probability of premature rupturing. Reproductive thermotolerance in tomato was characterized using three assays. *In vitro* pollen tube burst rates were measured in replicates of eight per accession, at both 26°C and 34°C, for eight diverse tomato accessions. Pollen tube length (at 26 °C) for each of the eight selected accessions was measured in ImageJ, to determine if there is any correlation between pollen tube length and pollen tube burst rate. Pistil lengths of the of the same accessions were measured during the first day of flower opening to determine if pistil lengths influence pollen tube burst rate. While there was no significant correlation between pistil lengths and burst rates, burst rates were directly correlated with pollen tube lengths. Accessions with comparatively shorter pollen tubes resisted bursting under heat stress conditions, whereas accessions with longer pollen tubes burst at a higher rate. This research presents a direct benefit to the agriculture: identifying cultivars with short pollen tube lengths may aid in developing thermotolerant crops.

Acknowledgements

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Purpose

To identify thermotolerant tomato accessions based on reproductive trait quantification.

Introduction & Rationale

Population growth, coupled with increasing environmental temperatures, places humanity in an imminent crisis. Discovering heat-tolerant crop varieties has become of paramount importance. *S. lycopersicum* (var. *lycopersicum*), the commonly cultivated tomato variety for human consumption, is one of the most valuable vegetable crops and a widely used plant research model organism (Razifard et al., 2020). Successful pollination in tomatoes under acutely high temperature stress requires thermotolerant female and male components, both of which will be addressed in this research proposal (Palanivelu et al., 2011).

The flower pistil, comprised of stigma, style, and ovary, encompasses the female reproductive organ in plants. Herkogamy, the relative pistil-to-anther length, plays a significant role in whether the flower favors autogamy (self-pollination) or allogamy (cross-pollination) (Opedal, 2018). Flowers exhibiting exerted stigmas, where the pistil extends beyond the anther cone, favor outcrossing, whereas flowers with inserted stigmas favor autogamy due to the proximity of adjacent self-pollen (Shang et al., 2021). The transition from allogamy to autogamy is the most common shared evolutionary step in flowering plants (Pan et al., 2017). Agriculture favors autogamous species due to reproductive assurance since successful fruiting is not dependent on pollinators (Eckert, 2000).

The stamen, consisting of the anther, filament, and pollen, constitutes the male reproductive system. Pollen is deposited on the flower stigma, where it hydrates, germinates, and forms a pollen tube that penetrates downward through the extracellular matrix of the style. Once the pollen tube reaches the ovules, the tube bursts, releasing the sperm cells (Cheung, 1996). Pollen formation, especially at the unicellular-to-early bicellular development stages, is particularly susceptible to heat stress. In tomatoes specifically, heat stress causes impaired pollen development in the anthers during pollen mother cells to microspores development, causing a significant decrease in fruit set (Giorno et al., 2013). However, the effect of the heat stress on subsequent steps are poorly understood, a gap that is addressed in this proposal.

Previous research has shown that ethylene increases pollen heat-stress resistance. Ethylene increases reactive-oxygen-species scavengers, which upregulates proteins involved in

pollen cellular functions, thus maintaining pollen function during heat stress (Jegadeesan et al., 2018). Pollen from thermosensitive species may burst prematurely before reaching the ovules, precluding fertilization. Generally, heat stress resistance can be attained through an initial exposure to elevated temperature, so that the plant will have resistance against a usually lethal dose later (termed “acquired thermotolerance”) (Giorno et al., 2013). Conversely, the innate ability to resist heat stress is termed “basal thermotolerance” (Jegadeesan et al., 2018).

While all the above discussion has addressed pollen development, the focus of this research rests on the subsequent critical steps of pollen tube function in the style. To date, “our understanding of the genetic control of heat tolerance in tomatoes is still very limited.” (Gonzalo et al., 2020). Particularly, the mechanisms of heat tolerance during the pollen tube growth and fertilization phases of reproductive development are not well explored and understood. This study will characterize nearly 100 genetically diverse accessions of two tomato species to identify accessions with thermosensitive reproductive phenotypes – variations in stigma exertion and pollen tube growth. *S. lycopersicum* (SL) and *S. pimpinellifolium* (SP) are two common tomato species. SP, from coastal Peru, Ecuador, and Chile, is the closest relative of the domesticated tomato (SL) and is facultatively autogamous. Originating in that part of the world, SP is resilient to harsh temperatures and salinity (Razali et al., 2018).

This research aims to identify thermotolerant tomato species based on two key adaptations during reproduction – advantageous variations in pistil length and pollen tube growth. Tomatoes with shorter pistils are hypothesized to have greater thermotolerance because the pollen tubes are exposed to heat for a shorter period. In addition, shorter pollen tubes are hypothesized to correlate with shorter pistils, reducing the tube growth time and decreasing the probability of premature rupturing before fertilization.

Independent Variable: Tomato accession

Dependent Variables: Pollen tube burst rate under heat stress and pistil and pollen tube lengths

Controlled Variables

Use of the same technique for measuring pistil and pollen tube lengths, all plants were cultivated in the same greenhouse under the same watering, sunlight, and temperature conditions. Image measurements were blinded and randomized by not assigning accession identifiers to the images until all measurements were performed.

Extraneous Variables

Potential microscope image error, subjective scoring of traits during image analysis, potential flower variance within accessions caused by variable microenvironment.

Control Group

Analogous pollen tube burst tests were performed at elevated (34 °C) and control (26 °C) temperatures of the same accessions in equal replication to isolate heat stress as the independent variable.

Question

Do shorter pistils and pollen tubes correlate with increased reproductive thermotolerance in tomato?

Hypothesis

I hypothesize that tomato accessions with shorter pistils and pollen tubes will be more thermotolerant because the pollen tubes will be growing, and thus subject to premature bursting, for a shorter period.

Methodology

Part 1: Characterizing tomato pistil lengths

Identify two-hundred accessions of *S. lycopersicum* and *S. pimpinellifolium* with sequenced genomes and cultivate the accessions in duplicate until the plants begin flowering (previously completed by mentors Dr. Cedar Warman and Dr. Ravishankar Palanivelu). Allow flowers to fully open and use only flowers that have opened the day of measurement (day 0 flowers). For each accession, remove a fully opened flower and remove petals and part of the anther cone to reveal the pistil. Using calipers, measure the length of the pistil from the base of the ovary to the top of the stigma, and immediately record. Repeat this procedure for a total of twelve replicate flowers for each accession. Analyze and visualize the pistil lengths using R code.

Part 2: Visualizing pollen tube growth phenotypes

Obtain pollen tube images for the accessions with longest and shortest pistil lengths after two hours of growth, in replicates of eight for both heat stress (34 °C) and control (26 °C) temperatures (images obtained from Dr. Cedar Warman). **Blind and randomize images by not assigning accession identifiers to the images until all measurements were performed, to reduce bias.** Import the images to ImageJ and measure the lengths of ten random pollen tubes (in pixels) for each image from the 26 °C temperature conditions, beginning from the connection point of the tube to the pollen grain, extending to the tube tip. Determine the burst rate of each image from both temperature conditions using the CellCounter analysis tool. Mark each burst tube, successful tube, and unsure tube with labels, and record the totals for each condition.

Part 3: Data Analysis

Import all pistil and anther length measurement data into R Studio. Using these data, calculate the pistil lengths for each accession, and visualize the data using boxplot charts of the pistil lengths (pairwise) by accession. Identify accessions with long and short pistil lengths. Perform Kruskal-Wallis and Dunn's test to determine the statistical significance of differences between the means of the pistil lengths by accession. In a second analysis, import the pollen tube length and burst rate microscopy data into R Studio. Aggregate the pollen tube length and burst measurements for each accession and perform Kruskal-Wallis, Dunn's, linear model, and pairwise t-tests between temperature conditions for statistical significance. Visualize the pollen tube lengths and percent burst rates by accessions. Plot the pollen tube lengths against the burst rates (by accession) and correlate the pollen tube data to the pistil measurement data.

Results

Reproductive thermotolerance was characterized using three assays. First, the length of the pistils and anthers were measured using calipers during the first day of flower opening (pistil lengths: *Appendix I*). For this analysis, the researcher measured 247 flowers, combined with 1,030 flower measurements from identical conditions collected by Dr. Cedar Warman, a total of 1,277 flowers were measured. The data were filtered by complete cases, where each accession had at least twelve replicates, resulting in 1,176 flowers from 98 accessions. There was a noticeable variation in pistil length among these 98 accessions analyzed, with the average pistil ranging from 6.5mm to 11mm.

The accessions were reviewed to determine which of these 98 accessions also had *in vitro* grown pollen tube microscope image data at both standard temperatures (26 °C) and heat stress temperatures (34 °C) collected in replicates of eight for each temperature condition. The pollen tube burst rates of each image, at both 26°C and 34°C were measured by the researcher for all eight accessions (Figures 1, 2). In each of the 8 accessions analyzed, pollen tube burst at the heat stress temperature (34°C) was significantly higher than that at 26°C (Figure 1).

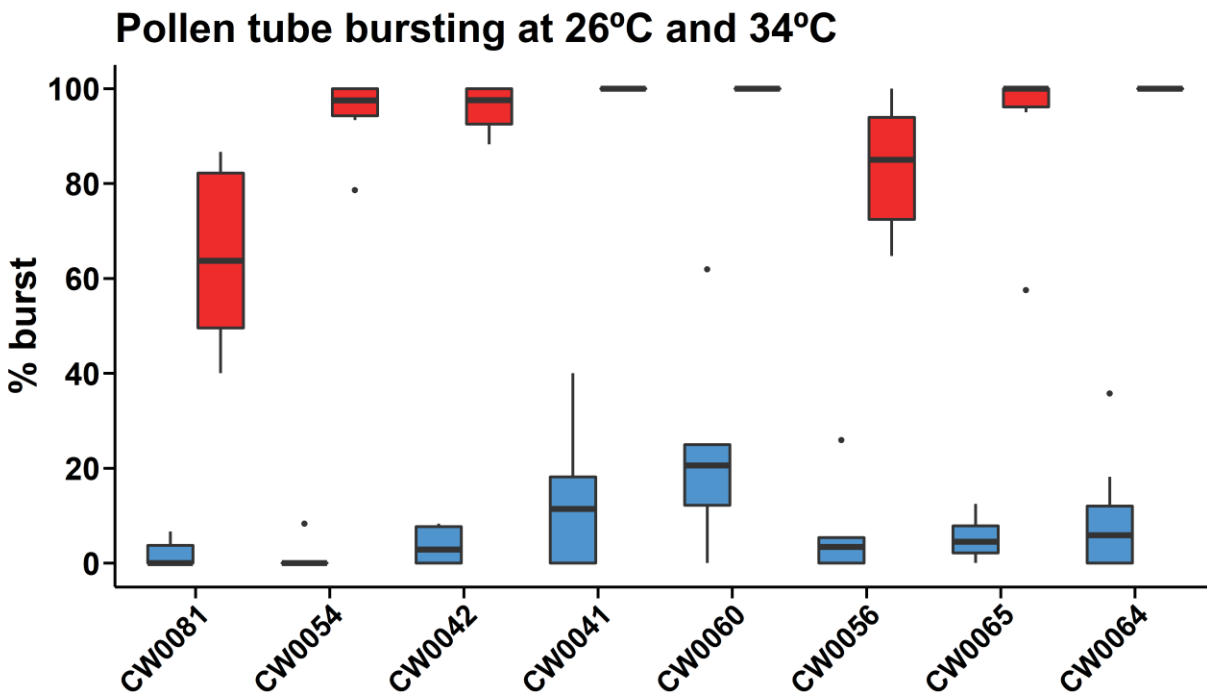


Figure 1. Pollen tube percent burst rates under both control temperature (26°C, blue) and heat stress (34°C, red) conditions, pairwise by accession.

Using Dunn's Test, accession CW0081 showed significantly reduced levels of pollen tube bursting at 34°C compared to that in the other seven accessions except CW0056 (p-value less than 0.05), and CW0056 had significantly lower pollen tube bursting than CW0081 and CW0065. **This indicates that there is a likely genetic locus or loci controlling this key characteristic.** If increased pollen tube bursting at 34 °C is significantly correlated with higher pollen growth potential, then pollen tubes that grew longer are perhaps more likely to burst at 34 °C and are thus unable to grow longer at high temperature stress.

To perform this correlation, the *in vitro* grown pollen tube length (at 26 °C) for each of the eight selected accessions was measured in ImageJ, to determine if there is any correlation between pollen tube length and burst rate. For this experiment, 80 pollen tubes were randomly measured from CW0081, 69 from CW0054, 80 from CW0042, 74 from CW0041, 76 from CW0060, 76 from CW0056, 80 from CW0065, and 76 from CW0064. In each accession, the number of pollen tubes measured were from images of at least eight replicates. Among the eight of accessions analyzed, there appeared to be a correlation between pollen tube length and pollen tube burst rate at 34 °C (Figures 2, 3).

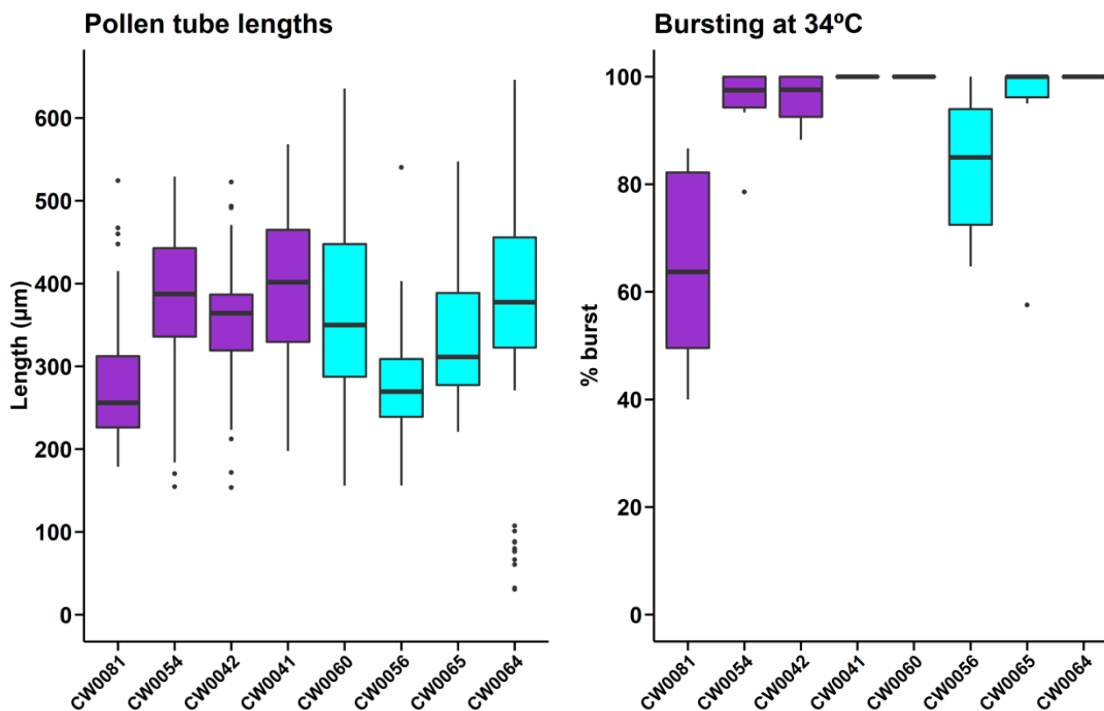


Figure 2. Selected accessions with short pistils (purple) and long pistils (cyan). Accessions are graphed by pistil length in millimeters (left), by pollen tube length in µm (middle), and by pollen tube burst rate percentage under 34 °C heat stress (right).

Accessions CW0081 and CW0056 were the accessions with the shortest pollen tube lengths, and the lowest pollen tube burst rate. (Figure 2, Table 4). To test these accessions for a potential correlation, the mean pollen tube burst rate (34 °C) was graphed against the mean pollen tube length (26 °C) (Figure 3).

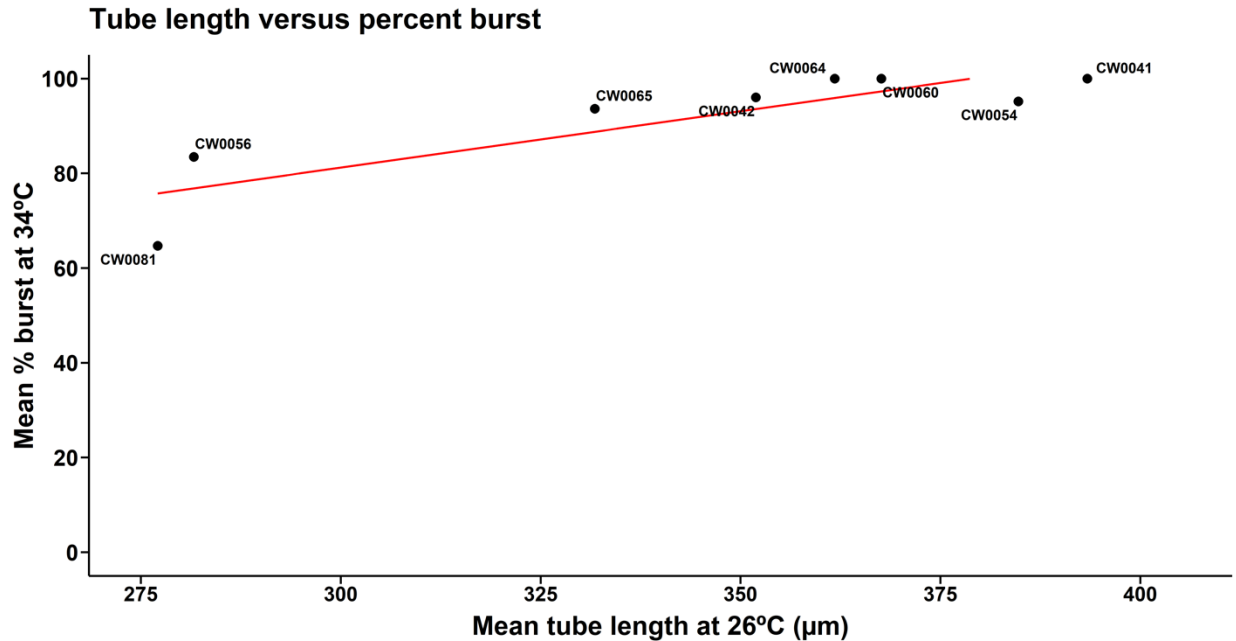


Figure 3. Average pollen tube length (µm) graphed against the pollen tube burst rate percentage. Points indicate accessions, labeled adjacent to the nearest point, graphed with trend line (red). Using a linear regression, the coefficient of equivalence had a p-value of 0.00615 and adjusted R² of 0.6964 indicating a noticeable correlation between tube burst rate and their growth potential (length at 26 °C).

Of the 46 accessions for which image data was also available, images of the accessions with the four shortest and four longest pistils were chosen for analysis. The accessions CW0081, CW0054, CW0042, and CW0041 represented the flowers with shorter pistils, and the accessions CW0060, CW0056, CW0065, and CW0064 exemplified longer pistils (Figure 4).

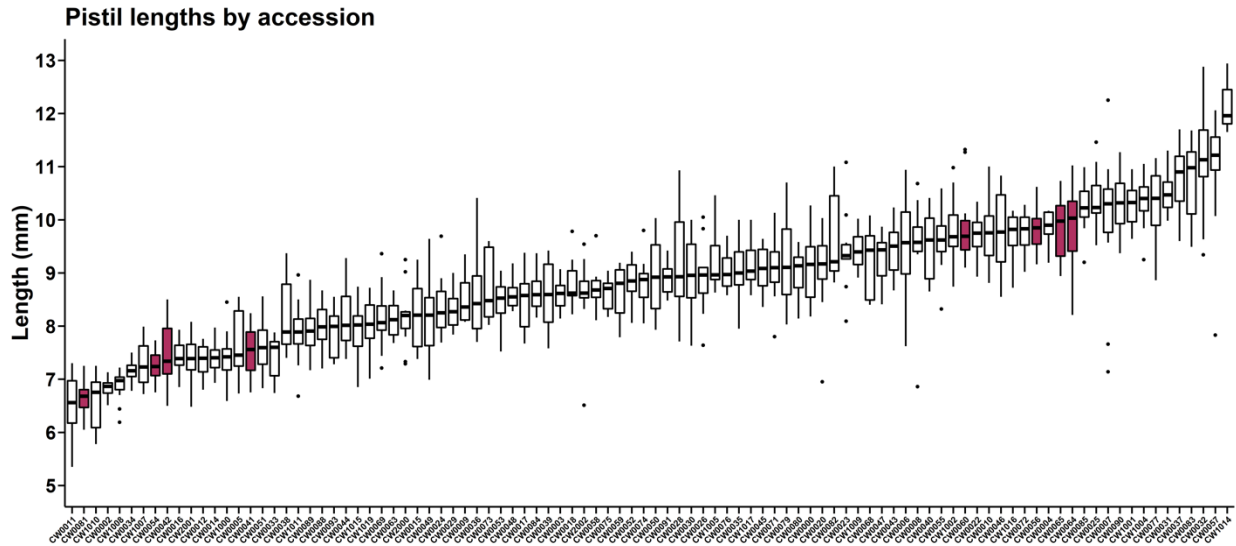


Figure 4. This graph illustrates the pistil lengths, in millimeters, of each measured tomato accession. The accessions with shortest and longest pistils, along with complete image data, are highlighted in maroon.

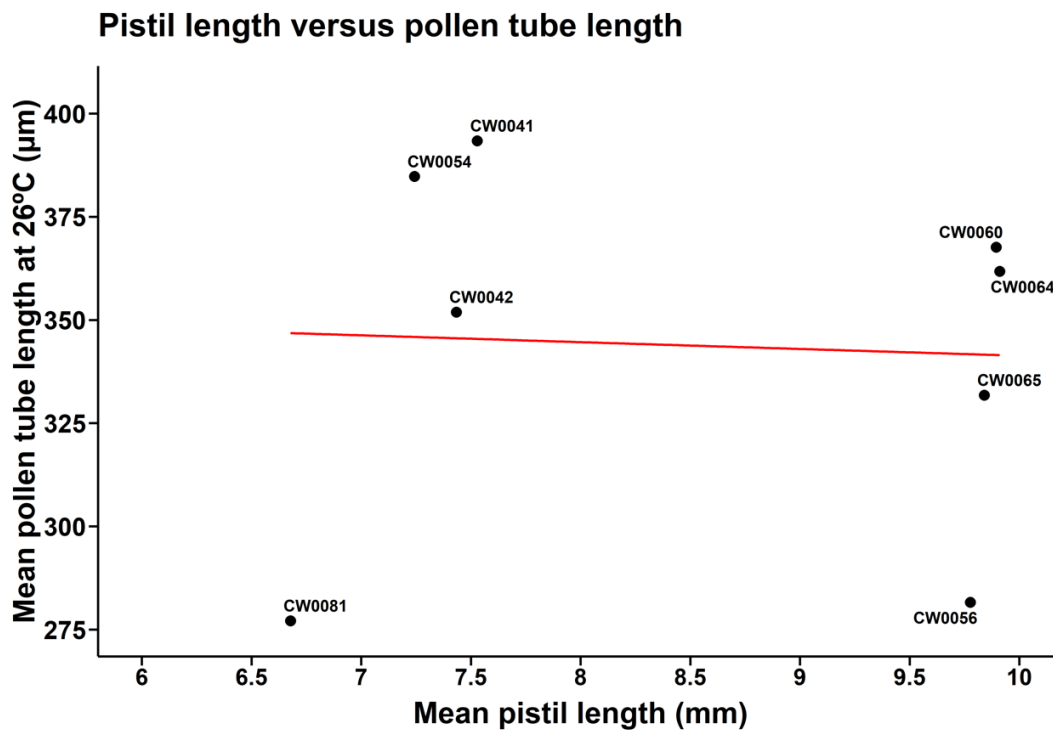


Figure 5. Mean pollen tube length (µm) graphed with mean pistil length (mm). Points indicate accessions, labeled adjacent to the nearest point, graphed with trend line (red).

The pistil lengths and pollen tube length linear regression had a p-value of 0.8996, and adjusted R^2 of -0.1633.

Since the data in this experiment were non-parametric (Appendix 1, Figures 7, 8), Kruskal-Wallis and Dunn's tests were used.

Pistil length: Kruskal-Wallis Test p-value: 9.729e-14

	CW0041	CW0042	CW0054	CW0056	CW0060	CW0064
CW0042	1					
CW0054	1	1				
CW0056	0.0097	0.0038	0.0012			
CW0060	0.0085	0.0033	0.001	1		
CW0064	0.0027	0.001	0.0003	1	1	
CW0065	0.0055	0.0021	0.0006	1	1	1
CW0081	0.6817	1	1	0	0	0

Table 1. Dunn's non-parametric statistical test. This table displays the adjusted p-values of pistil length comparisons between each accession. Values less than 0.05 are significant, indicated with green color mapping, whereas values greater than 0.05 are not significant (red).

Pollen Tube Length at 26 °C: Kruskal-Wallis Test p-value: 2.2e-16

	CW0041	CW0042	CW0054	CW0056	CW0060	CW0064
CW0042	0.2986					
CW0054	1	0.7697				
CW0056	0	0	0			
CW0060	0.4828	1	1	0		
CW0064	1	1	1	0	1	
CW0065	0.0011	1	0.0058	0.0068	0.9681	0.0745
CW0081	0	0	0	1	0	0

Table 2. Dunn's non-parametric statistical test. This table displays the adjusted p-values of pollen tube length comparisons between each accession at 26°C.

Pollen Tube Control Temperature Burst 26°C: Kruskal-Wallis Test p-value: 0.01512

	CW0041	CW0042	CW0054	CW0056	CW0060	CW0064
CW0042	1					
CW0054	0.2037	1				
CW0056	1	1	1			
CW0060	1	0.32	0.0048	0.3085		
CW0064	1	1	0.6316	1	1	
CW0065	1	1	1	1	0.9146	1
CW0081	0.7934	1	1	1	0.0327	1

Table 3. Dunn’s Test. Adjusted p-values of pollen tube burst rates at 26 °C control temperature.

Pollen Tube Heat Stress Burst 34°C: Kruskal-Wallis Test p-value: 2.551e-6

	CW0041	CW0042	CW0054	CW0056	CW0060	CW0064
CW0042	1					
CW0054	1	1				
CW0056	0.0258	1	1			
CW0060	1	1	1	0.0258		
CW0064	1	1	1	0.0258	1	
CW0065	1	1	1	0.8912	1	1
CW0081	0.0001	0.0334	0.0379	1	0.0001	0.0001

Table 4. Dunn’s Test. Adjusted p-values of pollen tube burst rates at 34 °C heat-stress.

Discussion

In this study, eight tomato accessions were quantified for pistil length, pollen tube length, and pollen tube burst rates under control and elevated temperatures. After performing pistil length analysis, and acquiring pollen tube image data, the accessions with the four shortest pistils are CW0081, CW0054, CW0042, and CW0041, and the accessions with the four longest pistils are CW0060, CW0056, CW0065, and CW0064. Kruskal-Wallis and Dunn's statistical tests were performed on the pollen tube length and burst rate under heat stress data, resulting in Kruskal-Wallis p-values of $2.2e-16$ for the length and 0.1783 for the burst data.

There was a significant correlation between pollen tube length and burst rate. Of the eight accessions, CW0081 and CW0056 had significantly shorter pollen tube lengths than the remaining six accessions (Figure 4, Table 2). The average pollen tube length was compared to average pollen tube burst rate using a linear regression. The pollen tube lengths were related to pollen tube burst rate, confirmed using a p-value (0.00615) and R^2 value (0.6964), **indicating a strong statistical significance between pollen tube length and pistil lengths.**

Pollen tube lengths significantly varied between accessions, but this variation was not significantly correlated to pistil length using a linear modeling p-value (Figure 5).

Using histograms as a normality check (Appendix I), there was some evidence that the data in this experiment were non-normal. Accordingly, non-parametric tests were utilized. First, a Kruskal-Wallis test was used to determine if there was any significant variance between any of the replicates. After a p-value of less than 0.05 was achieved, a Dunn's test was performed to determine which comparison(s) was significant. These procedures were repeated four times: for pistil lengths, pollen tube lengths, and burst rates under 26 °C and 34 °C temperatures. To ensure that there was a significant difference between the control and heat stress temperature burst rates, pairwise t-tests were used. All accession tests resulted in p-values less than 0.0001, indicating a significant difference in burst rates.

The result that pistil length is not correlated to burst rate was unexpected. This study relied on the principle that pollen tubes can grow independently from their corresponding pistil.

Kinase enzymes in tomato pistils, particularly *LePRK2*, have been shown to stabilize pollen tube growth through the extracellular matrix of the stigma and style tissues, and downregulation of this enzyme leads to shorter pollen tube lengths (Palanivelu et. al., 2011). Without the guidance of the pistil, it is likely that the *in vitro* pollen tubes in this study were unable to achieve their *in vivo* lengths. **Drawing a correlation between *in vivo* pistil lengths and *in vitro* pollen tube lengths proved inconclusive, however the pollen tubes lengths varied by accession, indicative of some phenotypic correlation.**

A pollen tube, and corresponding pollen grain, encompass a single male gametophyte. As the pollen tube develops, the cell forms several cytological “zones”. Throughout the length of the tube are several endoplasmic reticulum zones responsible for mRNA translation, however at the very tip, the tube contains a high number of vesicles, which serve as cell-wall precursors (Mascarenhas, 1993). Due to the sensitivity of the cell wall formation, bursting events are most likely to occur at the tip of the tube, whereupon the tube releases its sperm cells into the flower ovary. Pollen tube burst was tested as a potential rationale for why reproductive success was lower at higher stress temperature. If pollen tubes bursts prematurely, the sperm is not delivered to the ovary, and seed and fruit development are not initiated at optimal level. **Shorter pollen tubes grow at a slower rate, and accordingly face a decreased chance of premature bursting.**

The pistil lengths measured in this experiment reflect data from several recent studies. Shang and others (2021) performed a GWAS study on various *Solanum* species and did not notice a trend in pistil lengths between wild-type *S. lycopersicum* accessions, but improved accessions had shorter pistils than wild-type accessions. As a stress response, tomatoes may facultatively exert their pistils in response to high temperatures, mediated in part by *ToFZY2* and *SIAMI* genes which produce the growth hormone auxin in the pistils (Pan et. al, 2018). **This provides evidence that pistil lengths may fluctuate, based on abiotic stresses.**

To improve this protocol, an algorithm to track pollen tubes from microscope image data would allow for a greater quantity of images to be analyzed, such as the system proposed by Brau and others (2011).

Conclusion

I accept my hypothesis that tomato accessions with shorter pollen tubes are more thermotolerant and reject my hypothesis that tomato accessions with shorter pistils are more thermotolerant. Pollen tube length is significantly correlated to pollen tube burst rate under elevated temperatures, and burst rates are not significantly correlated to pistil length.

Practical Application

This research discovered a coefficient describing pollen tube length relative to pollen tube burst rate. Accessions with comparatively shorter pistils resisted bursting under heat stress conditions, whereas accessions with longer pistils burst at a higher rate. **This research represents a direct benefit to the agriculture.** Identifying additional species with short pollen tube lengths (and the gene(s) causing this phenotype) **may aid in developing thermotolerant crops, a necessity with rising global temperatures.**

Future Research

Future research will involve expanding this work to all 200 accessions and perform GWAS analysis to identify genetic loci that underlie key pollen tube growth and behavior characteristics. Another goal would be to determine genes causing short pollen tube lengths which will include mRNA tissue analysis and CRISPR procedures. Pollen tube lengths are mediated in part by genetic loci. Up-expressed pollen genes in accessions with short pollen tubes may be knocked out, to determine their role in tube length, and then expressed in species with long pollen tubes in attempt to replicate the short pollen tube phenotype. This may allow several crop species to be able to survive even under elevated temperatures.

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Appendix I

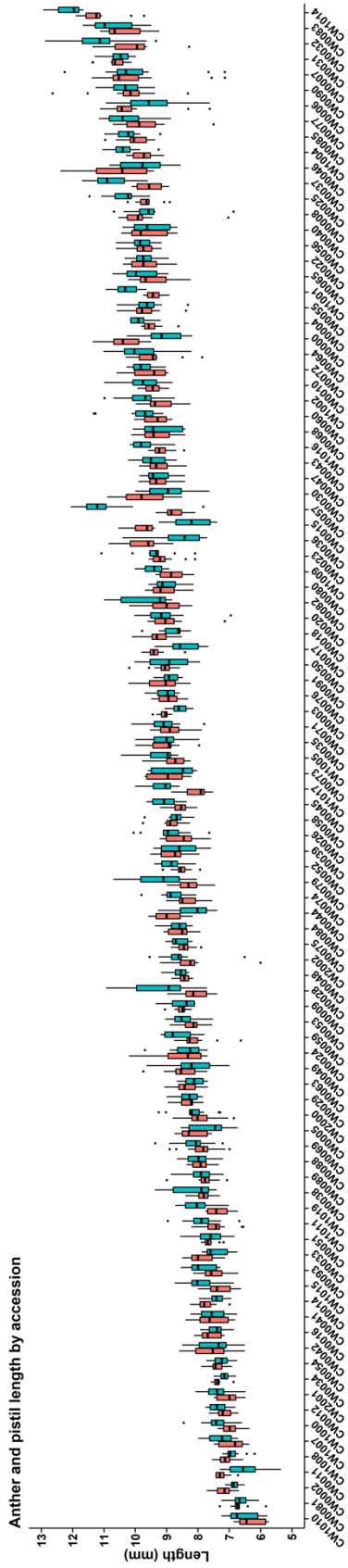


Figure 6. Anther and pistil lengths pairwise by accession number (CWXXXXX). Red plots are anther lengths, blue are pistil lengths. 98 accessions are shown, representing 1,176 flowers. Lengths are in millimeters. The boxplots represent median, second and third quartiles, and distribution of the measurements for each accession.

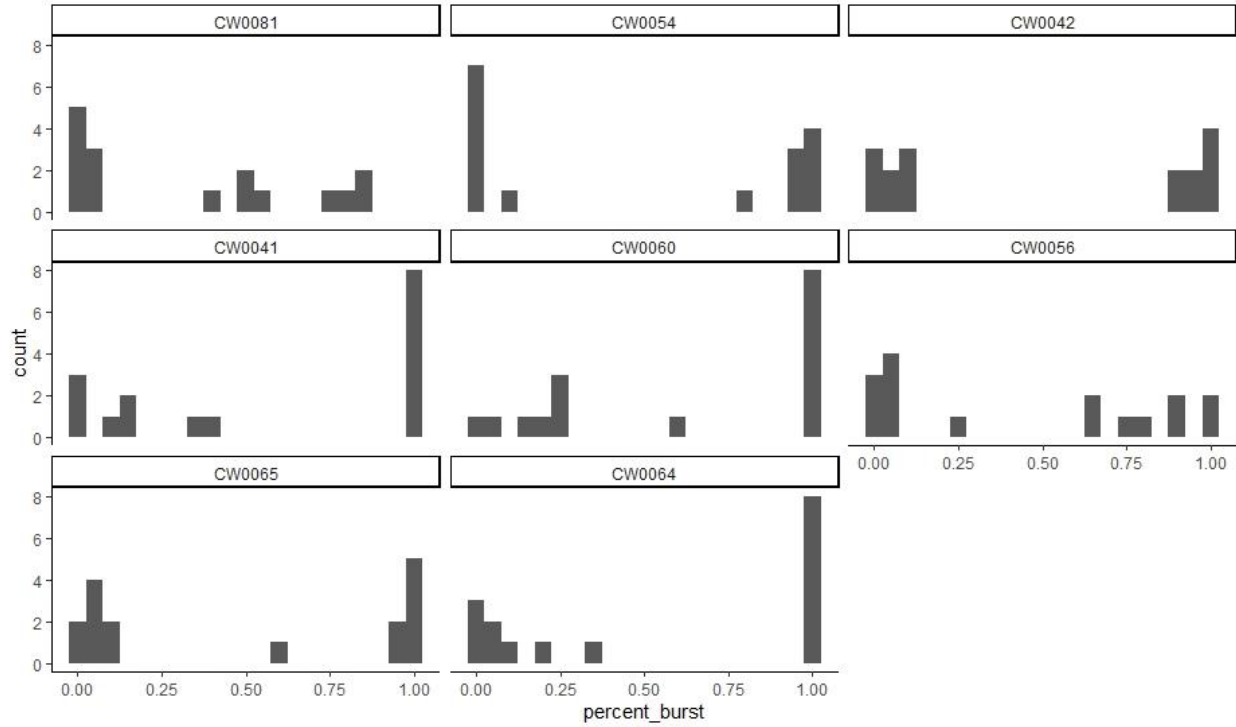


Figure 7. Histograms of burst measurements by accession. Since these data are not parametric, Kruskal-Wallis and Dunn's tests were used.

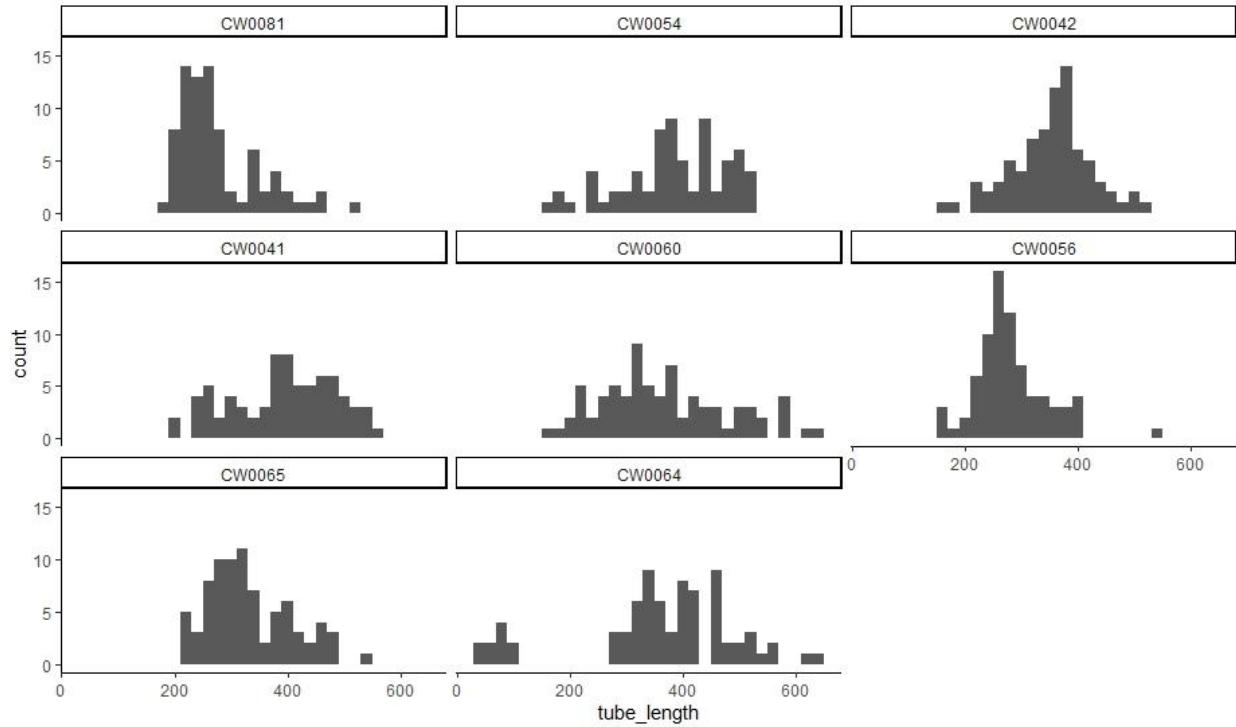


Figure 8. Histograms of pollen tube measurements by accession. Again, since these data are not parametric, Kruskal-Wallis and Dunn's tests were used.