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Characterization of tomato pollen thermotolerance mechanisms – for improving pollen quality and yield under heat-stress

Submitted to the Chief Scientist of The Ministry of Agriculture – Agricultural Biotechnology Committee

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תקציר

טמפרטורות גבוהות פוגעות ביבול ובאיכות הפרי של גידולים רבים ביניהם עגבנייה, כשהפגיעה בחנטה והיבול נובעת בעיקר בשל פגיעה באבקה המתפתחת. אולם, המידע הקיים על מנגנוני עמידות באבקה הינו מוגבל ביותר. מטרות התוכנית: 1. אפיון תפקיד מסלול האתילן בתגובה- ובעמידות-לחום של גרגרי אבקה של עגבנייה. 2. אפיון יחסי הגומלין בין מסלול האתילן ומסלולים אחרים בתגובה- ובעמידות-לחום של גרגרי האבקה. תוצאות ושיטות: על מנת לאפיין תפקיד אתילן בתגובה ובעמידות גרגרי האבקה לחום, נעשה שימוש בשתי מערכות ניסוי: (I) בחינת ההשפעה של טיפול בצמחים/בתפרחות בחומר המשחרר אתילן ('אתפון') (II) שימוש במוטנטים הפגועים בגנים במסלול החישה והולכת הסיגנל של אתילן. נמצא שטיפול מקדים באתפון מעלה באופן משמעותי את איכות האבקה בצמחים שנחשפו לעקת-חום, בהשוואה לצמחים הלא-מטופלים. כמו כן, נמצא שצמחי *EPI*, מוטנט המייצר אתילן ביתר, הראו את איכות האבקה הגבוהה ביותר, כמו גם המספר הגבוה ביותר של זרעים לפרי, ואחוז פירות עם זרעים בעקבות עקת-חום, בהשוואה לצמחי WT והמוטנטים האחרים שנבדקו (*NOR*, *RIN* ,*NR*). בנוסף, מצאנו שגרגרי אבקה מייצרים אתילן, ומראים ביטוי של גנים במסלול הביוסינתיזה, החישה, הולכת הסיגנל, והתגובה לאתילן. המערכת שביססנו במהלך פרויקט זה שימשה לזיהוי מנגנונים המעורבים בתגובת גרגרי האבקה לחום, ומנגנונים המעורבים בעמידות לחום המתווכת ע"י אתילן, זאת ע"י שימוש בגישה פרוטיאומית. התוצאות הראו שעקת-חום פוגעת במנגנונים חיוניים להתפתחות האבקה, כולל הומאוסטאזיס של חלבונים. טיפול מקדים באתפון שינה את פרוטאום האבקה בתגובה לחום, כך שהפך דומה לפרוטאום האבקה שהתקבל בתנאי ביקורת, כולל עלייה בביטוי חלבונים המבקרים תרגום, וחלבונים של מעגל ה-TCA. מעבר לכך, טיפול באתפון גרם לעלייה במנגנוני הגנה המתמודדים עם נזקי חמצון. בנוסף, מצאנו שלגרגרי האבקה היכולת לעמידות נרכשת לחום, וננקטה גישה טרנסקריפטומית על מנת לזהות המנגנונים המעורבים. מסקנות: בוססה מעורבות אתילן בעמידות גרגרי אבקה של עגבנייה לחום, וזוהו מנגנוני עמידות-לחום המופעלים ע"י טיפול באתפון. נמצאה חפיפה בין מנגנונים אלה למנגנונים המעורבים בעמידות נרכשת לחום, כולל מעורבות חלבוני עקת-חום, אנטיאוקסידאנטים, ופקטורים המעורבים בתרגום.

<u>מעריכים ממולצים לבדיקת הדוח המדעי</u>

1 שמעון מאיר

2 כראות עיניכם

3

הצהרת החוקר הראשי:

הממצאים בדו"ח זה הינם תוצאות ניסויים.

הניסויים מהווים המלצות לחקלאים : לא

חתימת החוקר  **תאריך 2/01/17**

Background

Exposure to higher than optimal temperatures, reduces yield and decreases the quality of many crops including cereals, grain legumes and vegetable crops [1-6]. In tomato, production is limited by high temperature conditions, and this problem becomes crucial since a significant proportion of production is achieved in greenhouses (in order to avoid insect-transmitted virus infections), where the daily mean temperatures are high, especially during the warmer seasons. Short waves of high temperatures may also be detrimental. We have demonstrated in tomato that developing pollen grains are the most sensitive to both mild chronic and short-term acute heat-stress (HS) conditions [7-11]. Heat-tolerant tomato genotypes (exhibiting higher yield under HS) produced larger numbers of high quality pollen grains under HS, compared to all tested heat-sensitive genotypes [8]. However, the molecular basis for pollen grains' heat-stress-response (HSR) and pollen heat-tolerance (thermotolerance) is poorly understood [12-15]. Using two high throughput transcriptomic approaches: cDNA AFLP and the Affymetrix GeneChip® Tomato Genome Array, we have identified genes that are involved in pollen HSR and may contribute to pollen thermotolerance [11]. Contradicting previous notion, that pollen is unable to mount a 'significant' HSR, our results revealed high HS regulation of members of the heat shock protein gene family, in addition to the HS transcription factors A2 (HSFA2) and HSFA3, as well as the reactive oxygen species (ROS) scavenger ascorbate peroxidase (APX) and factors other than the classical HS-responsive genes [11]. Remarkably, our recently published data indicated HS induction of several ethylene-responsive genes in developing tomato pollen grains, including ethylene-responsive gene 5 (ER5), ER21, LeJERF1, ER24 as well as the gene coding 1-aminocyclopropane-1- carboxylic acid (ACC) synthase (ACS), the enzyme involved in ethylene biosynthesis, (exhibiting 4-, 63-, 9-, 156- and 6-fold induction following HS [11]), pointing to the involvement of ethylene in pollen HSR.

At the level of vegetative tissues, evidence for the involvement of ethylene in plant thermotolerance, was brought by numerous studies. For example, pretreatment of a cool-season grass (*Agrostis stolonifera var. palustris*) with ACC (a precursor of ethylene), prior to exposure of plants to HS, increased heat tolerance [16]. Similarly, ACC, when added exogenously, was shown to protect *Arabidopsis* against heat-induced oxidative damage [17]. Larkindale et al. [18] showed that the *Arabidopsis* ethylene signaling mutants, *ein2* and *etr1*, are defective in basal thermotolerance. However, there is no data available on the involvement of ethylene in pollen HS response and thermotolerance. A short description of ethylene biosynthesis and signaling pathway components is given in Appendix A, together with a scheme (Appendix A, Scheme 1).

The major aim of the suggested research project was to characterize the role of ethylene in tomato pollen HSR and thermotolerance.

Objectives

1. Characterize the role of ethylene in tomato pollen HS response and thermotolerance.
2. Identify potential links between the ethylene pathway and other pathways in tomato pollen HS response and thermotolerance.

Main experiments and results

1. Characterize the role of ethylene in tomato pollen HS response and thermotolerance

(a) Establishing involvement of ethylene in pollen thermotolerance

I. Characterize the effect of exogenously added chemicals that modulate ethylene levels, on pollen thermotolerance

For testing the effect of ethylene and ethephon (an ethylene-releasing substance) on pollen quality, we have first calibrated ethylene concentrations to eliminate/minimize flower drop. The results are presented in Appendix A Fig. 1. The results indicate that the effect of ethylene on flower drop was genotype-dependent (Micro-Tom plants exhibiting higher sensitivity) and that concentrations in the range of 0.25 – 0.5 ppm cause close to zero flower drop and are thus suitable to be used for further experiments.

Exposing tomato (*cv. Micro-Tom*) plants to HS conditions (2 h at 50 ^{o}C) at pollen developmental stages A-4 to A-1 (4 to 1 days before flower opening/anthesis, respectively) caused 4.8- and 2.5-fold fold elevation in the number of non-viable pollen grains when HS was applied at A-4 and A-2 stages, respectively, while no significant elevation was observed when HS was applied at A-1 (Fig. 1 and Appendix A Fig. 2). Pre-treating the plants with ethephon, at both A-4 and A-2 stages, prevented this increase, resulting in 2.2 X 10^4 and 1.1 X 10^4 non-viable pollen grains per flower in pre-treated plants compared to 4.1 X 10^4 and 2.1 X 10^4 , respectively, in non-pretreated plants (Fig. 1A and Appendix A Fig. 2). The population of viable pollen grains consisted of germinating and non-germinating pollen grains. Pollen germination capacity was tested by incubating mature pollen grains in germination solution as described in [11]. Heat stress caused a significant decrease in the number of germinating pollen grains at all tested flower developmental stages (Fig.1, C and D, Appendix A Fig. 2, C and D). For all tested flower developmental stages (A-4, A-3, A-2, A-1), pre-treating the plants with ethephon resulted in an increase in the number of germinating pollen grains following HS exposure. Highest, and significant increase was observed at the earlier

developmental stages A-4 and A-3: At A-4, 95% and 49% reduction in the number of germinating pollen grains was observed in non-treated and ethephon-treated plants, respectively, following HS, resulting in 10-fold higher number of germinating pollen grains in the ethephon pre-treated plants (0.2 X 10^4 and 2.3 X 10^4 germinating pollen grains per flower, respectively; Fig. 1C). At A-3, 92% and 46% reduction was observed in non-treated and ethephon-treated plants, respectively, resulting in more than 8-fold higher number of germinating pollen grains in the ethephon pre-treated plants (0.3 X 10^4 and 2.7 X 10^4 germinating pollen grains per flower, respectively; Fig. 1D). Taken together, the results indicate that pre-treating the plants with ethephon caused a significant increase in the number of germinating pollen grains and a significant decrease in the number of non-viable pollen grains following exposure to HS conditions, pointing to the involvement of ethylene in developing pollen grains (DPGs) thermotolerance. This effect was, however, stage-specific, with the earlier stages tested (A-4 and A-3; polarized microspore and early bicellular pollen stages) being more sensitive to both HS and ethephon treatments. It should be noted that neither HS nor ethephon applications caused a change in the leaf or the whole plant phenotype (data not shown).

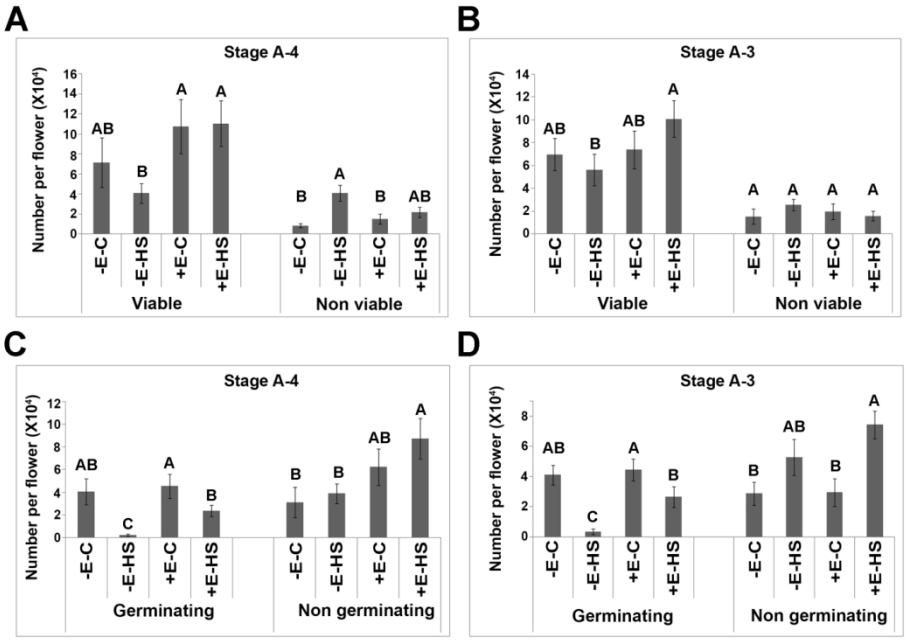


Figure 1. Effect of ethephon pretreatment on tomato pollen quality following exposure of the plants (Micro-Tom) to short-term heat stress (HS). Plants were either pre-treated with ethephon (+E-C, +E-HS; soaked in a solution of 1 ppm ethephon for 18 h before applying either control or HS conditions) or not pretreated (-E-C, -E-HS; soaked in water). HS - 2 h 50 ^{o}C, applied at 4 (*A, C*) and 3 days (*B, D*) before flower opening (stages A-4 and A-3, respectively). Mature pollen grains were collected and pollen quality determined. Data are presented as mean values ± SE (n = 3 biological replicates) of number of Viable, Non viable (*A, B*), Germinating and Non germinating (*C, D*) pollen grains per flower (each replicate being an average of pollen derived from 8 flowers collected from different plants). C – control conditions; 2 h at 25 ^{o}C. In each pollen quality category bars with different letters are significantly different by multiple comparison Tukey's HSD test (α = 0.05).

II. Use of tomato ethylene signaling mutants for testing the effect on pollen HS response and thermotolerance

In order to further substantiate the involvement of ethylene in pollen thermotolerance and point to specific signaling components that may be involved, we have used tomato mutants, mutated in different signaling pathways genes, as detailed below. The following four mutants (a gift from Dr. Lazaro, Brazil), in the background of the tomato cultivar Micro-Tom (small plants, having a short life cycle, and producing high number of flowers [19]) were used: *(i)* ***NR*** **–** *Never ripe*, a tomato mutant defective in the ethylene receptor (ETR3), a semidominant ethylene receptor mutant [20] - ethylene insensitive. (ii) ***RIN*** – mutation at the *RIN* (ripening inhibitor) gene - ethylene independent (iii) ***NOR*** – *Non ripening* gene mutant - ethylene insensitive. (iv) ***EPI*** – *epinasty* - ethylene over-producer.

Plants were grown in two temperature-controlled greenhouses at The Volcani Center in Bet Dagan, Israel, under natural light conditions (day length of 13.5 – 14 h) and day/night temperatures of 26/23 ± 2 ^{o}C (designated control conditions) for up to one month. These environmental conditions have been found to ensure high pollen quality. Then, one of the greenhouses was set to day/night temperatures of 35/23 ± 2 ^{o}C (designated mild chronic HS conditions; MCHS) and the plants were kept, in both greenhouses, for two additional months, producing flowers and fruits. Mature pollen grains were sampled from plants growing under the two temperature regimes after at least 14 days of exposure to MCHS, to determine the number of viable (stained purple) and non-viable (stained green) pollen grains, using Alexander dye [21]. The number of germinating pollen grains was calculated as well, as described [8]. Data were collected during the summer seasons of 2014 and 2015. Results of pollen quality derived from flowers of WT compared to *NR*, *RIN*, *NOR* and *EPI* mutants, grown under control and MCHS conditions, are summarized in Fig. 2. The results indicate that for WT as well as for all tested mutant lines, the number of non-viable pollen grains was relatively low and was hardly affected by the MCHS conditions applied (35/23 ± 2 ^{o}C day/night temperatures). The main effect of MCHS was the significant reduction in the number of germinating pollen grains in WT as well as in all tested mutants except *EPI* (Fig. 2). Thus, *EPI* stands out as exhibiting thermotolernance at the level of pollen quality.

III. Use of tomato ethylene signaling mutants for testing the effect on seeded fruits production under HS conditions

Wild type and mutant plants were grown under 'control' and MCHS conditions as detailed above, let to set fruits, and the numbers of fruits produced per plant and average fruit weight were measured during two seasons and the results are presented in Appendix A, Fig. 3. The results indicate large variation in fruit number in plants of all tested lines, under both control and MCHS conditions (Appendix A, Fig. 3A). *EPI* plants showed the highest average fruit

weight, which was not affected by HS, while WT and all other tested mutant plants exhibited significantly reduced fruit weight upon exposure to HS conditions (Appendix A, Fig. 3B).

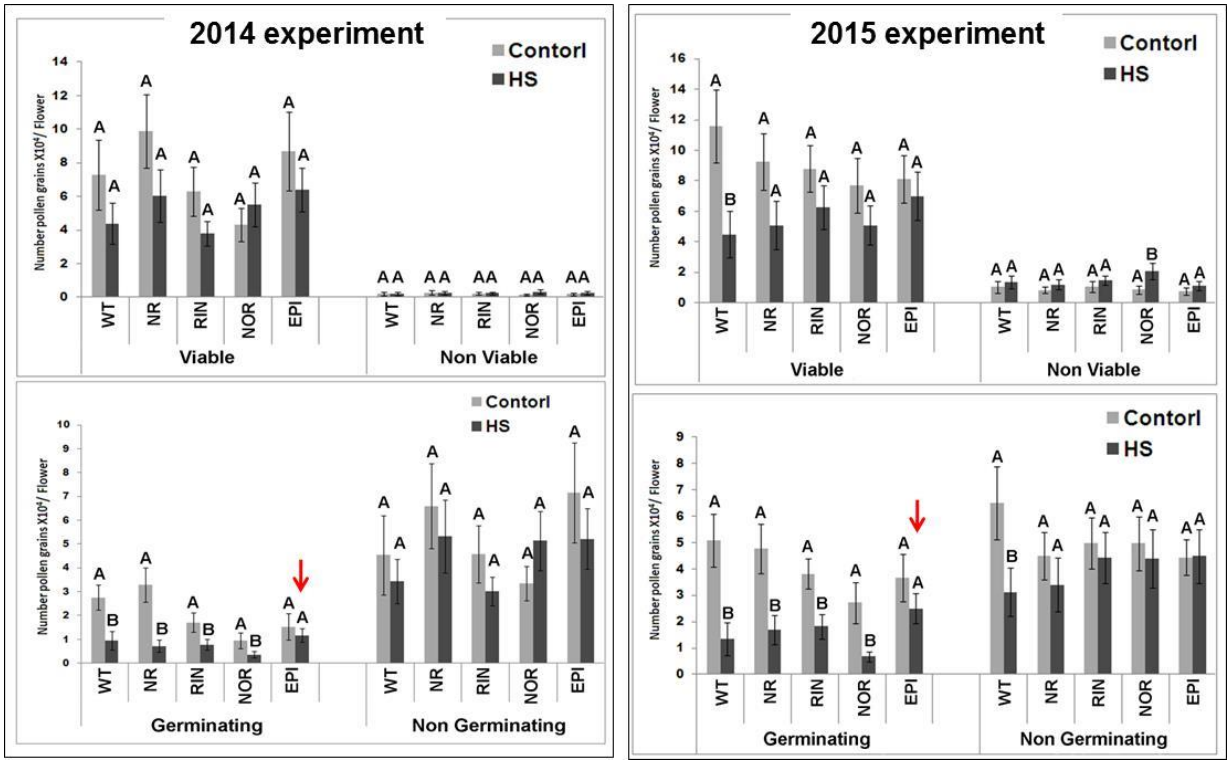


Figure 2. The effect of mild chronic heat-stress conditions (HS; 35/23 ± 2 ^{o}C day/night temperatures) on pollen quality of WT and ethylene signaling mutant tomato (Micro-Tom) plants. The data are presented as mean values ± SE (n = 4 biological replicates). Each biological replicate consisted of pollen grains derived from 8 flowers. Bars with different letters represent significantly different values by Tukey's HSD test analysis ($p \leq 0.05$). Control - 26/23 ± 2 ^{o}C day/night temperatures.

The effect of HS conditions on numbers of seeded fruits per plant, as well as the number of seeds per fruit, was analyzed during two seasons and the combined results are presented in Fig. 3A and B, respectively. The results indicate that *EPI*, the ethylene over-producer mutant, exhibited highest percentage of seeded fruits per plant (Fig. 3A) and highest number of seeds per fruit (Fig. 3B) under HS conditions. A representative picture of the fruits of the different lines is given in Picture 1. It is interesting to note that in *RIN* mutant plants percentage of seeded fruits per plant following HS exposure did not differ significantly from the number under control conditions (Fig. 3A). The results thus point to a beneficial effect of both *EPI* and *RIN* mutations on tomato seeded fruits production under HS. Seed weight was also affected by HS, and *EPI* was again outstanding, exhibiting increased average seed weight under HS (Appendix A, Fig. 4).

(b) Do pollen grains have the capacity for ethylene production?

I. Measuring ethylene in pollen and in supporting sporophytic tissues

To the best of our knowledge, there is no data available regarding, both, the potential of pollen grains for ethylene production and the existence of ethylene biosynthesis and

signaling pathways in microspores and pollen grains, as well as the presence of unique and pollen-specific components of these pathways. As a first step, we have analyzed the amount of ethylene produced by tomato flowers/flower buds, anther tissues and isolated pollen grains at developmental stages A-3, A-1 and A (3, 1 and 0 days before flower opening/mature pollen grains stage, respectively). Flowers and anthers (4-6, weighing 500 mg and 100 mg, respectively), at the respective developmental stages were placed in a 15 and 2 mL sealed tubes, respectively, for the indicated time and ethylene concentration produced was analyzed by withdrawing a 2-mL gas sample with a hypodermic syringe and injecting it into a gas chromatograph (Varian, Palo Alto, CA) equipped with an activated-alumina column and a flame-ionization detector. For the analyses of ethylene production by pollen grains, pollen, at the respective developmental stages, were isolated in germination solution without added sucrose as described [11] using at least 60 flowers per sample.

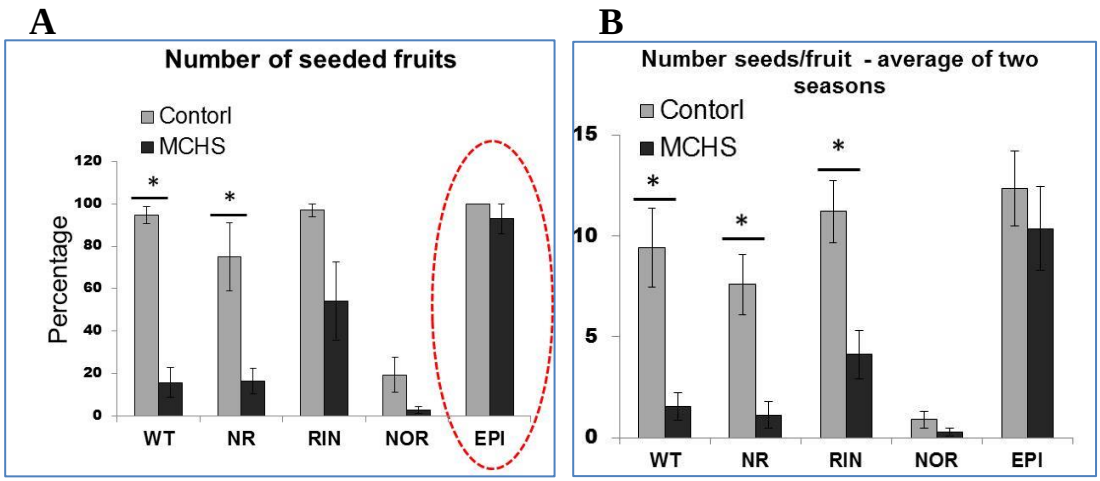
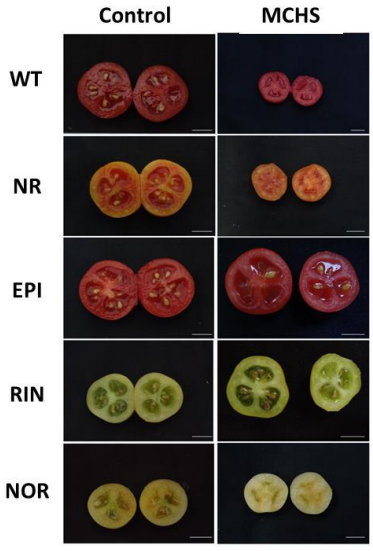


Figure 3. The effect of heat- stress conditions on percentage of seeded fruits per plant (A) and number of seeds per fruit (B) in WT and tomato ethylene signaling mutants. Tomato (MicroTom) plants were grown at either control 26/23 ± 2 ^{o}C or mild chronic heat-stress conditions (MCHS) 35/23 ± 2 ^{o}C day/night temperatures. Fruits were collected from 30 plants (during years 2014 and 2015). Number of fruits tested for seed presence in each mutant and wild type lines was 40 and 20 fruits in each of the two seasons for (A) and (B), respectively. Data are presented as mean values ± SE (n = 3 biological replicates). (*) - represent significantly different values by student t test analysis ($p \leq 0.05$).



Picture 1. Representative pictures of fruits produced by WT and ethylene signaling mutant plants exposed to either control or MCHS conditions. Control - 26/23 ± 2 ^{o}C day/night temperatures. MCHS - 35/23 ± 2 ^{o}C day/night temperatures.

Isolated pollen grains were kept in a minimum volume of the remaining solution, in 2 mL sealed tubes for the indicated time and ethylene was measured as described above. Ethylene measurements were done following exposing the flowers to either HS (2 h 45 ^{o}C) or control (2 h 25 ^{o}C) temperature treatments. The effect of these HS conditions, applied at the indicated flower/pollen developmental stages, on pollen quality was recorded using a detached flower system of tomato *cv. 3017* (Appendix A, Fig. 5). Pollen quality analyses indicate that HS affected the number of germinating pollen grains at all developmental stages tested (causing more than 80% reduction) and caused elevated number of non-viable pollen grains when applied at the earlier developmental stage, A-3 (Appendix A, Fig. 4).

The results presented in Fig. 4 show ethylene production by flowers, isolated anthers, and isolated pollen grains, at three developmental stages. The different flower developmental stages tested were characterized with respect to pollen development stages as shown in Appendix A, Picture 1.

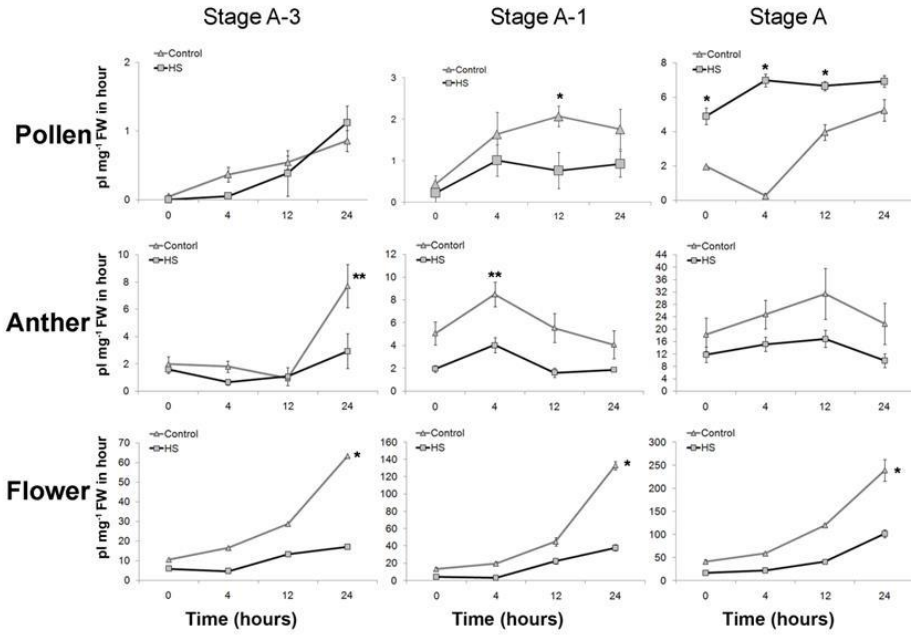


Figure 4. Ethylene production by tomato flowers, anthers and pollen grains and the effect of heat-stress. Data represent the amount of ethylene accumulated during the indicated time. HS -2 h 45 ^{o}C (represented by squares) – was applied at the indicated flower/pollen developmental stages and samples were collected immediately after the treatment. Control – 2 h 25 ^{o}C (represented by triangles). Pollen grains were kept in minimum amount of germination solution with no sucrose. Three biological replicates were used for each analysis. * and ** - significantly differen,t in HS vs. control, by student t test analysis at $p \leq 0.05$ and $p \leq 0.01$, respectively. For pollen isolation, > 60 flowers for each biological replicate were used.

In all tested tissues, including isolated pollen grains, ethylene production could be detected during a 24 h incubation time. Highest ethylene levels were produced, at the mature flower stage (A), by all tissues examined; 238, 22 and 5 pl/mg/h for flowers, anthers, and pollen grains, respectively (Fig. 4). The capacity for ethylene production increased during flower/pollen maturation by 3.8-, 2.5- and 4-fold for flower, anther and pollen, respectively. Heat stress application (2 h 45 ^{o}C as detailed above) caused a reduction in ethylene production by flowers and by anther tissues at all tested developmental stages (Fig. 4). For

example, 10- and 3-fold reduction by HS was observed at 'A' stage for flowers and anthers, respectively (Fig. 4). Interestingly, isolated pollen grains exhibited a different behavior, showing HS-reduced production at 'A-1' stage and a > 1.4-fold increased production at 'A'. The results thus indicate that developing and mature pollen grains have the capacity of ethylene production and that HS affects this capacity in a developmental-stage-specific way.

II. Ethylene biosynthesis and signaling genes in tomato pollen grains

Aiming at identifying the presence of pollen ethylene-biosynthesis and -signaling pathway components, their characterization, and their HS response, we have analyzed the expression of ethylene pathway genes in the following tissues of tomato *cv. 3017*; microspores/pollen grains at developmental stages of 3, 1 and 0 days before flower opening (A-3, A-1 and A, respectively) as well as anther tissues without pollen grains (the tissue supporting pollen development). As a positive control, we have used leaf, mature green fruit and mature red fruit tissues. The results of expression at the RNA level of ethylene-biosynthesis, -signaling and -responsive genes, in leaf and fruit (green-fruit & ripe-fruit) tissues are given in Appendix A, Fig. 6. We have analyzed the expression of eight ACC synthase (*ACS*) genes (*ACS1-ACS8*) and one *ACS* homologue detected in pollen transcriptomic data as will be detailed below (*ACS-h - Solyc03g007070*), as well as the expression of five ACC oxidase (*ACO*) genes (*ACO1-5*) and one pollen expressed ACO homologue (*ACO-h - Solyc06g060070*). ACS8 and ACO2-3 showed low expression (Appendix A, Fig. 6). Regarding the ethylene signaling genes, we have analyzed expression of six ethylene receptor genes, *ETR1-6*, four *CTR* genes, *CTR1-4* and one additional homologue (*CTR-h - Solyc08g065250*), four *EIL* genes, *EIL1-4*, *EIN2*, *EBF1-2*, *ETO1*, *ERN1* and five ethylene responsive genes (*ERF1*, *ERF3*, *ERF-h*, *JERF1*, *JERF3*,*ER21;* see Appendix A scheme 1 for a scheme of tomato ethylene biosynthesis and signaling). Heat stress application caused a general down-regulation in the expression of tested *ACS* and *ACO* biosynthesis genes, except for *ACS5* that showed HS-up-regulation in green fruit (Appendix A, Fig. 6). Heat stress-down-regulation was observed for all ethylene receptor genes tested, while *CTR2* and *CTR3* exhibited HS-up-regulation in at least one of the tested tissues (green fruit, Appendix A, Fig. 6). *EIL1* and *EIL4* exhibited HS-down-regulation while *EIN2* exhibited HS-up-regulated expression in leaf tissue and down-regulation in ripe fruit.

Following calibration of the system, gene expression profiles of ethylene-biosynthesis and –signaling gens were tested in anther-wall tissues (known to respond to ethylene, involving ethylene receptor genes, especially during dehiscence; [22-23] and in isolated pollen grains (for which there was no data available) and results are presented in Appendix A Figs. 7-8.

The results indicate that ethylene biosynthesis pathway is functioning in pollen at mRNA level: The isoforms of *ACS* and *ACO* genes are differentially expressed and exhibit a stage-specific expression during pollen development: *ACS1*, *3*, *4*, *7* and *ACO1, 3*, *4* are expressed at 'A-3' stage, *ACS1*, *4*, *7*, *8* and *ACO1*, *4* are expressed at 'A-1' stage and *ACS2*, *7*, *8* and *ACO1*, *4* are expressed at 'A' stage. Thus *ACO3* and *ACS2* are found to be expressed exclusively at A-3 and A stages, respectively. Heat stress caused a significant up-regulation of *ACS3* expression (>9-fold) at both A-3 and A-1 stages, raising its expression at A-1 to detectable levels (Appendix A, Fig. 7), and down-regulation in *ACS4* expression (>10-fold) at A-3 and A-1 stages as well as down-regulation in *ACO4* expression (>6-fold) at A-3 and A developmental stages.

It is interesting to note that anther tissues expressed *ACS2*, *3* and *ACO1*, *4*, *5* during all tested developmental stages (A-3, A-1, A), expressing *ACS4* at A-3 and A-1 stages and did not show detectable expression of *ACS1*, *7*, *8* and *ACO3* detected in isolated pollen grains (Appendix A, Fig. 7). Heat stress caused down-regulation in expression of *ACS2* (>2.4-fold) at A-3 and A stages and, similar to pollen, down-regulation of *ACO4* expression (>2.3-fold) at A-3 and A stages (Appendix A, Fig. 7).

Regarding the identification of ethylene signaling pathway components and their expression analyses in anthers compared to pollen, less components were expressed by isolated pollen grains as compared to anther-wall tissues at the tested developmental stages: In anthers, six receptor genes (*ETR1-6*) were expressed while in pollen only three (*ETR3-5*); Four and three *CTR* genes (*CTR1-4* and *CTR2-4*, respectively), and four and one *EIL* genes (*EIL1-4* and *EIL3*, respectively) were expressed in anthers and pollen grains, respectively (Appendix A, Fig. 8). In both anther and pollen *EIN2*, *EBF1-2*, *ETO1* and *ERN1* were found to be expressed. Thus, pollen grains were found to express the main components of the ethylene signaling pathway. Interestingly, all pollen ethylene signaling genes (except *ERN1*) exhibited down-regulated expression in mature pollen grains (A) compared to the earlier developmental stages tested (A-3 and A-1), suggesting that the pathway is active (at least at the mRNA level) during pollen maturation phase. A similar pattern of expression was observed for the tomato anther wall tissue, except for *ETR6* and *EIL2*, that in addition to *ERN1*, exhibited relatively high expression in mature pollen grains compared to the earlier developmental stages tested (Appendix A, Fig. 8). The application of HS caused, in both pollen and anther tissues down-regulation in gene expression of specific ethylene signaling components and this effect was more pronounced in pollen. For example, in pollen, at developmental stage A-3, more than 6-fold reduction in the expression *ETR3*, *ETR4*, *EIN2* and *EIL3* by HS was observed (Appendix A, Fig. 8).

Regarding ethylene responsive genes, similar results were observed for pollen and anther tissues, expressing ER21 (ethylene-responsive heat shock protein cognate 70) and ER24 (multiprotein bridging factor 1) during all tested developmental stages, exhibiting high HS-up-regulation of more than 17- and 100-fold for ER21 and ER24, respectively (Appendix A, Fig. 9). These data are currently being summarized into a manuscript.

(c) Identify pollen thermotolerance mechanisms

I. Ethylene-mediated pollen thermotolerance mechanisms - a proteomic approach

A proteomic approach was undertaken, to unravel the mechanisms underlying pollen heat-stress response and ethylene-mediated pollen thermotolerance in developing pollen grains. Developing pollen grains were harvested from flowers at developmental stages of four and three days before flower opening (A-4 and A-3, respectively, exhibiting HS-sensitivity and responsiveness to ethephon pretreatment, as detailed in Fig. 1) derived from Micro-Tom tomato plants, grown at day/night temperatures of 28/22 ± 2 ^{o}C, that were exposed to short-term HS conditions (50 ^{o}C for 2 h) following treatment with either water or ethephon. Pollen grains were harvested immediately after HS application, using at least 40 flowers per each developmental stage, and pooled for proteomic analysis. The pooled pollen sample was found to consist of 11% unicellular microspores, 44% polarized microspores and 45% early bicellular pollen grains, as calculated by counting 200 pollen grains derived from 10 plants in each of three biological replicates (Appendix A, Fig. 10). This population is referred to as developing pollen grains (DPGs). The following four DPGs samples were used for proteomic analysis: DPGs derived from plants maintained at 25 ^{o}C (control conditions; P-C), DPGs derived from ethephon-pre-treated plants that were maintained at 25 ^{o}C (P-E-C), DPGs derived from heat-stressed plants (P-HS), DPGs derived from ethephon-pre-treated plants that were exposed to HS (P-E-HS). Three biological replicates of each treatment were analyzed and separated into two fractions via SDS-PAGE prior to tryptic digestion and LC-MS/MS analysis similar to [24-25].

In total, 1355 proteins were identified from all four pollen samples (P-C, P-E-C, P-HS, P-E-HS). Protein abundances were quantified by peptide count and an NSAF normalization strategy [26]. All raw data, as well as the lists of all identified proteins will be deposited into a public database upon acceptance of our manuscript for publication. Principal components analysis (PCA) of the protein NSAF scores was performed using COVAIN [27]. A PCA of the pollen proteins revealed that the samples could be separated into two major groups (Fig. 5), with one including only the HS-exposed DPGs (P-HS), and the other including DPGs derived from the following three treatment groups: P-C, P-E-C and P-E-HS. Thus, the separation in the PCA points to the HS-specificity of the P-HS proteome. Moreover, grouping together of

the P-C, P-E-C and P-E-HS samples in the PCA, points to alterations in the heat-stressed DPGs' proteome following ethephon pre-treatment, causing it to re-group with the 'control' (non-heat-stressed) samples.

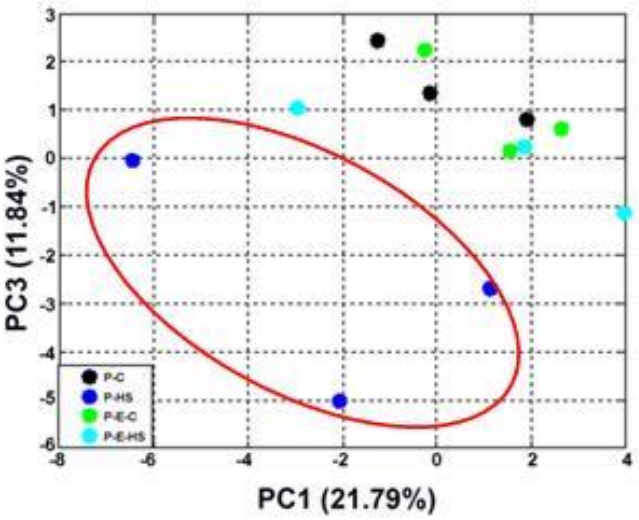


Figure 5. Principal component analysis (PCA) of pollen protein NSAF scores. For each treatment group, 3 biological replicates were used.

This separation in the PCA of the P-HS samples (dark blue dots), on one hand, and grouping of the P-E-HS samples (light blue dots) with the P-C and P-E-C samples, on the other hand, point to high responsiveness of DPGs to HS, and to a 'neutralizing/protective' effect of the ethephon pre-treatment at the proteome level, respectively. The data thus serve as a valuable tool for looking into the HS effect on the DPGs' proteome in order to come up with changes associated with the significant reduction in pollen quality as well as looking into potential protective mechanisms activated by ethephon pre-treatment.

In order to further group the pollen proteins according to their presence in the different treatment groups, the NSAF scores were normalized for each protein and the proteins were clustered using the k means algorithm with k = 30 [27], considering proteins present in all three replicates. In the following we discuss only selected clusters which showed a treatment-specific profile. Four clusters were found which include proteins displaying decreased or increased levels in P-HS relative to all other treatments (clusters 8 and 15, 26, 25, respectively; Fig. 6). Proteins displaying reduced levels in P-HS (cluster 8, Fig. 6A) included several 40S and 60S ribosomal proteins (RPs; Solyc06g007570, Solyc10g006070, Solyc06g083180, Solyc08g006040, Solyc07g065170 and Solyc07g009330, Solyc10g078960, Solyc06g073300, respectively), tryptophanyl-tRNA synthetase (Solyc08g074410) and eukaryotic translation initiation factor (Solyc02g089070) involved in translation, pointing to an effect of the applied HS on degradation (or on reduction in expression/translation/synthesis) of specific proteins, causing reduced levels and loss of components of the translational machinery. Since pre-treatment with ethephon prior to HS exposure (P-E-HS sample) caused more than 1.6-fold increased levels (compared to P-HS)

of all these proteins (data not shown and will be included in the paper submitted for publication as supplementary data), it is hypothesized that ethylene may have a role in protecting DPGs' translation machinery.

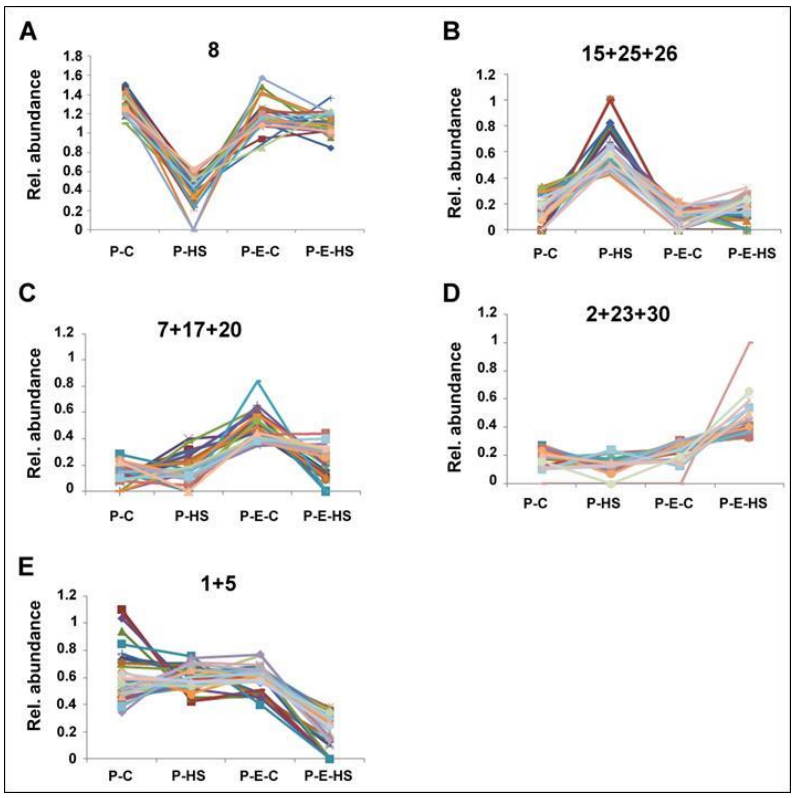


Figure 6. Cluster analysis. Relative abundance of proteins in a selection of groups obtained via k means clustering. NSAF scores were averaged over three biological replicates and normalized for each protein to represent their proportion of the total abundance over all four treatments The proteins constituting each of the individual, *A,* or combined, *B – E,* clusters presented in *A – E* will be included in a supplemental Table in the publication, once accepted. P-C (pollen derived from plants maintained at control conditions), P-HS (pollen derived from plants exposed to HS conditions), P-E-C (pollen derived from plants pretreated with 1 ppm ethephon followed by maintaining the plants at control conditions), P-E-HS (pollen derived from plants pretreated with 1 ppm ethephon followed by exposing the plants to HS conditions).

Several RPs, including subunits of 40S and 60S RPs, showed HS-up-regulation compared to control conditions (Fig. 6B), pointing to differential regulation of pollen RPs under HS. It is interesting to note that almost half of the HS-down-regulated proteins in cluster 8 (46%; Fig. 6A) were mitochondrial, including, for example, prohibitin (Solyc05g051510) which was suggested to play a role in mitochondrial biogenesis and protection against oxidative stress [28]. Indeed, mitochondrial proper functioning is essential for pollen development and abnormal development of the male reproductive tissues leading to male sterility was found to correlate with modifications of mitochondrial functioning [29]. A subset of proteins grouped in clusters 7, 17 and 20 (Fig. 6C) was expressed predominantly in ethephon-pre-treated DPGs, and these proteins might take part in the maintenance of important cellular functions as well as the regulation of pollen protective mechanisms, activating them prior to exposure of DPGs to high temperatures and enabling maintenance of high pollen quality under HS. This group included proteins involved in carbohydrate metabolism and glycolysis, reproduction, cell redox homeostasis and response to abiotic stimulus. One example of proteins in this cluster (Fig. 6C) include glutaredoxin family protein (Solyc06g067960), protein disulfide isomerase (Solyc05g056400) and glutathione disulfide reductase (Solyc09g091840), involved in redox homeostasis [30]. Redox regulation and signaling have come into sight as crucial

mechanisms able to manage critical stages during sexual plant reproduction [31]. Furthermore, it has been previously demonstrated that HS is accompanied by oxidative stress and that exogenous application of ACC protected *Arabidopsis* against heat-induced oxidative damage, thus pointing to a role for ethylene in activating mechanisms/genes that alleviate oxidative damage. Glutaredoxins are small heat-stable oxidoreductases that transfer electrons from glutathione (GSH) to oxidized cysteine residues, shown to contribute to protein integrity and regulation and function in antioxidant defense [32]. Glutathione-disulfide reductase (GSR) catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH), which is a critical molecule in resisting oxidative stress and maintaining the reducing environment of the cell. Protein-disulfide isomerase is an oxidoreductase enzyme that belongs to the thioredoxin superfamily. Taken together, these three proteins, up-regulated by ethylene, may play a role in preventing harmful effect of reactive oxygen species (ROS) in DPGs under HS conditions. Additional proteins, grouped in clusters 2, 23 and 30 (Fig. 5D), expressed predominantly in ethephon-pre-treated DPGs following HS application, correlate with higher pollen quality under HS. This group included proteins that regulate translation, carbohydrate metabolism and response to misfolded proteins. One interesting example is a group of enzymes that play a role in carbohydrate metabolism and may be involved in signaling, regulating carbon assimilation and sugar status in plants, such as trehalose-6-phosphate synthase (Solyc10g007950, puatative; [33]. The subset of proteins grouped in clusters 5 and 1 show reduced levels of specific proteins in the ethephon-pretreated sample following HS-exposure, relative to all other treatments (Fig. 5E), and contain proteins that function in cellular catabolic processes, including protein degradation by specific proteases and by the proteasomal ubiquitin-dependent protein catabolic process. In particular, aspartic proteinase (Solyc07g051850) as well as several components of the proteasome including SKP1 (Solyc01g111640; a core component of the SCF complex, a major type of E3 ubiquitin ligase catalyzing the last step in ubiquitin-mediated protein degradation pathway [34] and two 26S proteasome regulatory subunits (Solyc05g01g111640, Solyc05g018570) exhibited reduced abundance. Taken together, the results point to an important role of protein homeostasis- regulation, during pollen development, in enabling pollen proper functioning.

This proteomic data was summarized into a manuscript and will be submitted soon.

2. <u>Identify potential links between the ethylene pathway and other pathways in tomato pollen HS response and thermotolerance</u>

(a) Developing pollen grains have the capacity for acquired thermotolerance

In order to determine whether tomato pollen grains have the capacity for acquired thermotolerance (ATT), plants (*cv. Hazera 3017*) were exposed to mild HS (1 h at 38 ^{o}C, followed by 1 h recovery at 25 ^{o}C) prior to exposure to short-term acute HS conditions (AHS; 2 h at 50 ^{o}C), and the effect on pollen quality was tested. Exposing the plants to HS at the post-meiotic maturing pollen stage (3 days before flower opening, corresponding to early binucleate pollen stage) caused more than 50% reduction in the number of germinating pollen grains (Fig. 7). Exposure of the plants to ATT conditions, prior to exposing them to AHS, resulted in a more than two-fold increase in the number of germinating pollen grains (11.9 x 10^4 per flower) which was comparable to the average number of germinating pollen grains under control optimal conditions (11.6 x 10^4 per flower).

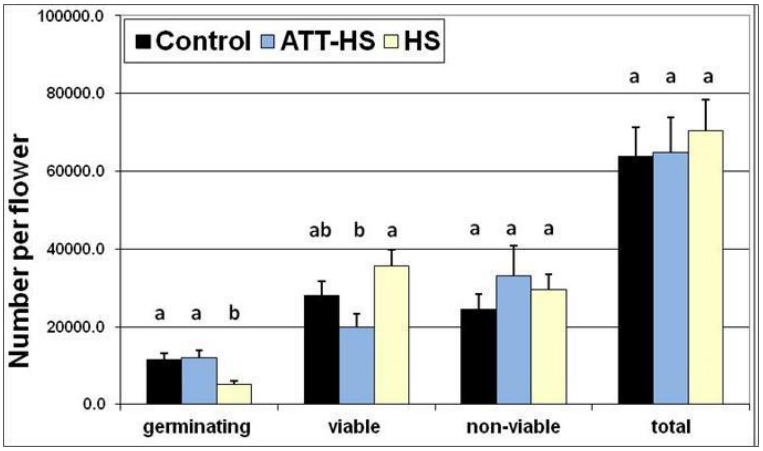


Figure 7. Effect of pre-treating tomato plants with mild heat-stress on pollen quality following exposure of the plants to acute heat stress. Tomato plants (*cv. Hazera* 3017) were pretreated, at maturing (post-meiotic) pollen grains stage, by exposure of the plants to mild HS (ATT conditions; 1 h exposure to 38 ^{o}C followed by 1 h recovery at 25 ^{o}C), before acute HS application (2 h exposure to 50 ^{o}C). Mature pollen grains were collected from plants that were maintained at 25 ^{o}C ('Control'), plants that were exposed to acute HS ('HS') and plants that were exposed to ATT conditions before acute HS application ('ATT-HS'), and pollen quality was determined. Data are presented as mean values ± SE (n = 24 biological replicates (flowers); collected in 3 experiments) of number of germinating, viable and non-viable pollen grains per flower. In each pollen quality category (germinating, black bars; viable, light blue bars; non-viable, yellow bars) bars with different letters are significantly different by multiple comparison Tukey's HSD test (p value < 0.05). Total number of pollen grains per flower is composed of number of germinating + viable + non-viable pollen grains.

The results indicate that ATT conditions, applied during tomato pollen development, can protect pollen functioning from HS damages, pointing to activation of pollen thermotolerance mechanisms/genes.

(b) Acquired thermotolerance-activated mechanisms – a transcriptomic approach.

Looking for cross-talk between ethylene-mediated and other pollen thermotolerance mechanisms.

The established ATT conditions were further used in order to identify pollen HS-protective mechanisms, by performing a detailed transcriptomic analysis of DPGs and mature pollen grains (MPGs) using the Illumina next-generation sequencing platform. The list of expressed transcripts in all four treatment groups (DPGs-C, DPGs-ATT, MPGs-C, MPGs-ATT is available as an excel file and can be added to this report in case requested). The

differentially expressed transcripts (p-value ≤1e-05) in DPGs_C versus DPGs_ATT were analyzed for GO-category enrichment relative to the pollen transcriptome using AgriGO and were found to contain 139, 53 and 69 significantly enriched (p-value <0.01) GO functional terms in the biology process (BP), molecular function and cellular component, main categories. For most GO terms, the majority of transcripts were found to be up-regulated following exposure of the plants to ATT conditions, constituting 99%, 89% and 99%, respectively, indicating general up-regulation in gene expression following ATT treatment. The BP category was found to be most informative and the results of 22 most-enriched BP GO terms are presented in Fig. 8.

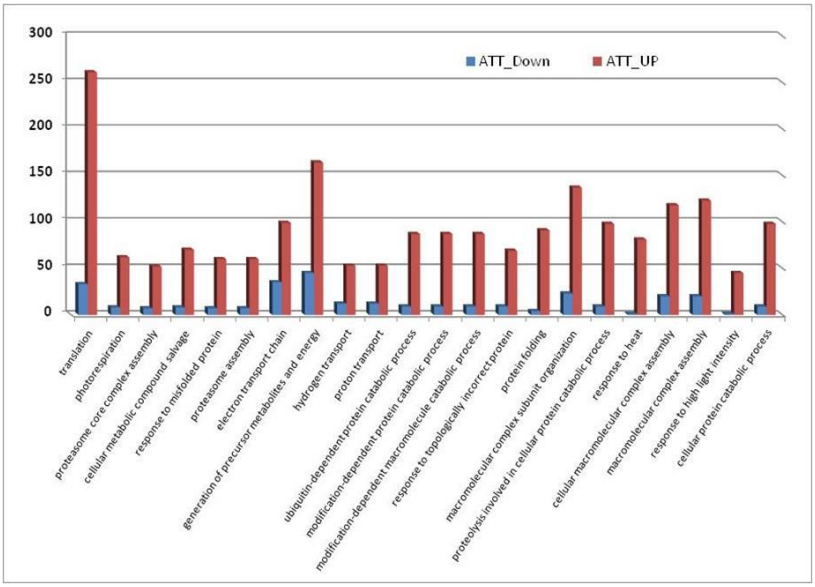


Figure 8. GO enrichment analysis of differentially expressed transcripts (p-value ≤1e-05) of developing pollen grains following acquired thermotolerance (ATT) treatment. Enrichment was done relative to the pollen trancriptome data and results of 22 most enriched GO terms (p-value <0.01) in the biological process category are presented.

The most enriched GO term (p-value 2.36e-19) was "translation" (GO:0006412, containing 33 down- and 261 up-regulated transcripts following ATT), including the child term "translational elongation" (GO:0006414, p-value 0.00089), pointing to a role for protein synthesis in the acclimation of maturing pollen grains to high temperatures. Up-regulation following ATT of a relatively large number of transcripts representing translation initiation factors (including eIF-4 and eIF-5) was also detected, in addition to up-regulation of several transcripts of ribosomal proteins and ribosomal protein homologues (including ribosomal protein L10). 'Protein folding' (GO:0006457; *p-value* 9.10e-08), 'response to heat' and 'response to high light intensity' (GO:0009642) were among these highly enriched GO categories (Fig. 8) indicating a role in pollen ATT. 'Response to stress' (GO:0006950) and child terms 'response to light intensity' (GO:0009642), 'response to reactive oxygen species' (GO:0000302), 'response to temperature stimulus' (GO:0009266), 'heat acclimation' (GO:0010286) and 'response to oxidative stress' (GO:0006979) were among the unique functions enriched in DPGs following ATT treatment.

The term 'response to stress' included 480 ATT-up-regulated transcripts and 66 ATT-down-regulated transcripts. 259 transcripts exhibited ≥2.5-fold higher read number in response to ATT treatment. Percentage of transcripts belonging to a given functional group for the 259 transcripts is presented in Appendix A, Fig. 11. Functional classification of these 259 ATT-upregulated transcripts indicates that the largest group (31.2%) consists of heat shock proteins (HSPs) and chaperones, including small HSPs, HSP70, HSP90 and HSP100 family members, other interesting groups are the 'anti-oxidants', containing ascorbate peroxidase, glutathione S-transferase, pyridoxal biosynthesis protein and peroxiredoxin, as well as 'translation', containing several subunits of eukaryotic translation initiation factors 3, 4 and 5. Regarding the group 'hormones', it contained indole-3-acetic acid-amido synthetase, allene oxide synthase, methyle jasmonate esterase and GID2, suggesting the potential involvement of auxin, jasmonate and gibberrellin in ATT, however, no genes related to ethylene were included in this group.

Discussion

During the project, the involvement of ethylene in maintaining tomato pollen quality under HS was established by using two main approaches: (1) Characterizing the effect of exogenously added ethephon, an ethylene releasing substance, on pollen thermotolerance
(2) Using tomato ethylene signaling mutants. Use of the first approach showed that pre-treating the plants with ethephon (an ethylene-releasing substance) caused a significant increase in the number of germinating pollen grains and a significant decrease in the number of non-viable pollen grains following exposure to HS conditions (Fig. 1), pointing to the involvement of ethylene in DPGs' thermotolerance. This effect was, however, stage-specific, with the earlier stages tested (A-4 and A-3; polarized microspore and early bicellular pollen stages) being more sensitive to both HS and ethephon treatments. Use of the second approach, demonstrated that the *epinasty* (*EPI*) mutant, which is an ethylene-over-producer, exhibited high pollen quality under MCHS conditions, as compared to WT and other ethylene signaling mutant plants tested (Fig. 2). In accordance with maintaining higher pollen quality under MCHS, all fruits (100%) produced by *EPI* plants contained seeds ('seeded fruits'), while WT, *NR* and *NOR* plants produced only 10%, 22.5% and 5% seeded fruits, respectively and the number of seeds per fruit under MCHS was also highest in *EPI* (Fig. 2). These results, establishing a role for ethylene in tomato pollen thermotolerance, highlight the potential of using treatment protocols for enabling better yield under HS conditions. Such treatments should be calibrated with respect to substance concentrations, mode and timing of application.

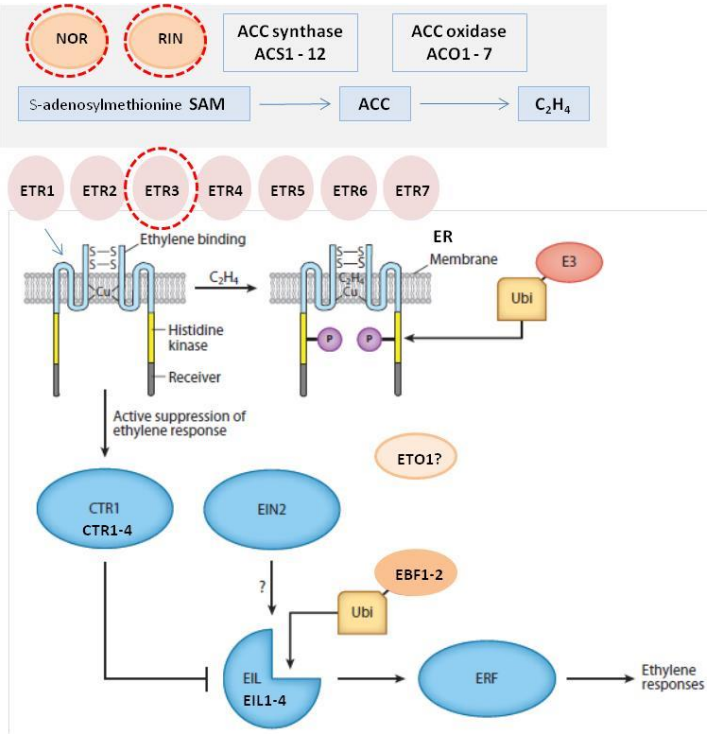
Another interesting and relevant question was whether tomato pollen have the capacity for ethylene production and whether DPGs contain components of the ethylene-biosynthesis, -signaling, and –responsive pathways. To the best of our knowledge, there is no data available regarding these issues. As a first step, we have analyzed the amount of ethylene produced by tomato flowers/flower buds, anther tissues and isolated pollen grains at developmental stages A-3, A-1 and A. Ethylene production could be detected in all tested tissues, including isolated pollen grains, showing elevated levels at the mature flower/pollen stage (Fig. 4). In pollen, HS was found to affect ethylene production in a developmental-stage-specific way, causing reduced production at 'A-1' stage and a > 1.4-fold increased production at 'A'. These results emphasize the differences between the various pollen developmental stages. In petunia, it was suggested that ethylene is important for germination [35], thus the HS-induced ethylene may increase germination capacity. Furthermore, we have demonstrated that pollen grain possess ethylene biosynthesis and signaling-gene-components that are actively expressed during development (Appendix A, Figs. 7 and 8, respectively). The results indicate that specific components are expressed in pollen as compared to the supporting anther tissue, pointing to developmental regulation during pollen development. The HS effect was mainly down-regulation of expression of biosynthesis and signaling pathway genes in both pollen and anther tissues. It should be noted that ACC synthase 3 exhibited significant HS-upregulation in developing pollen. Stresses such as cold, drought, and salt were shown previously to induce high levels of *ACS* transcripts, the level of which was reported to be associated with the amount of ethylene production [36-37]. Thus pollen ACS3 deserves further attention/studies with respect to its response to the environment and the effect on ethylene production. EBF1 and 2 were shown to be HS up-regulated in anthers at 'A' and 'A-2' stages, respectively. EBF (EIN3-binding F-box) proteins are known to negatively regulate the ethylene signaling pathway via mediating the degradation of EIN3/EIL proteins. They are found to have redundant function and their differential up-regulation in anthers following HS may point to alterations in their function at specific flower developmental stages in response to the environment. Thus, this system of pollen and anther maturation may be used for obtaining novel data on functional importance of the ethylene pathway & pathway components in the context of plant reproduction.

The established system during this project was further used in order to unravel mechanisms underlying pollen HS response and ethylene-mediated pollen thermotolerance, using a proteomic approach. The analysis revealed that heat-stress affected the developmental program of pollen, including protein homeostasis (components of the translational and degradation machinery), carbohydrate and energy metabolism. Ethephon-

pretreatment shifted the heat-stressed pollen proteome closer to the proteome under non-stressful conditions and resulted in higher abundance of proteins involved in protein synthesis, degradation, TCA cycle and RNA regulation. Furthermore, up-regulation of protective mechanisms against oxidative stress was observed following ethephon-treatment, including higher abundance of glutathione-disulfide reductase, glutaredoxin and protein disulfide isomerase. Redox regulation and signaling have come into sight as crucial mechanisms able to manage critical stages during sexual plant reproduction [31]. Furthermore, it has been previously demonstrated that HS is accompanied by oxidative stress and that exogenous application of ACC protected *Arabidopsis* against heat-induced oxidative damage, thus pointing to a role for ethylene in activating mechanisms/genes that alleviate oxidative damage. Glutaredoxins are small heat-stable oxidoreductases that transfer electrons from glutathione (GSH) to oxidized cysteine residues, shown to contribute to protein integrity and regulation and function in antioxidant defense [32]. Glutathione-disulfide reductase (GSR) catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH), which is a critical molecule in resisting oxidative stress and maintaining the reducing environment of the cell. Protein-disulfide isomerase is an oxidoreductase enzyme that belongs to the thioredoxin superfamily. Taken together, the results suggest that these three proteins, up-regulated by ethylene, may play a role in preventing harmful effects of reactive oxygen species (ROS) in DPGs under HS conditions.

Furthermore, we have demonstrated that, similar to vegetative tissues [18], developing tomato pollen grains have the capacity for acquiring thermotolerance. Namely, pre-treating plants/inflorescences with mild HS prior to exposing them to a more severe HS protected pollen quality (Fig. 7). A transcriptomic approach was undertaken to identify mechanisms activated by the ATT treatment, highlighting an overlap between the etephon- and ATT-activated mechanisms and pointing to a role for HSPs, ROS scavengers/anti-oxidants and translation initiation factors in pollen thermotolerance. The ATT-modulated pollen transcriptome did not show significant changes in the expression of ethylene-related genes, suggesting that ATT-induced thermotolerance does not function through the ethylene pathway. It is thus suggested that ATT and ethephon treatments may activate the same/similar regulatory cascade. Further research is necessary in order to identify such regulator/s and several candidates, such as MBF1, may be identified in our database of the DPGs ATT transcriptome.

Appendix A



Scheme 1. **Schematic representation of ethylene biosynthesis and signaling pathways in tomato**. The scheme was adopted from [38] and modified to include additional components, including the ethylene biosynthesis pathway. The mutants used in this project are marked on the scheme by a red circle.

Ethylene is derived from the amino acid methionine. The methionine is converted to S-adenosyl L-methionine (SAM) by the enzyme SAM synthetase. The SAM is then converted to ACC and 50-deoxy-50-methylthioadenosine (MTA) by **ACC-synthase** (ACS). The conversion of SAM to ACC is the rate limiting step in the biosynthetic pathway. The **ACC-oxidase** (ACO) catalyzes the conversion of ACC to ethylene. There are at least 12 *ACS* and 7 *ACO* genes in the tomato genome with specific temporal and spatial expression patterns [44]. Main ethylene biosynthesis and signaling components are illustrated in Figure 1. The **ethylene receptors** are endoplasmic reticulum–associated, integral membrane proteins, with protein kinase activities [[38] and references therein]. Genetic analysis in tomato and *Arabidopsis* has shown that the receptors act as negative regulators of the ethylene response pathway. In the absence of the hormone, receptors actively suppress ethylene responses. Upon ethylene binding, that suppression is removed and the response occurs. The only single receptor mutations that have observable phenotypes confer dominant ethylene insensitivity. One of the earliest known tomato fruit ripening mutants is the dominant *Never-ripe* (*Nr*) mutation. *Nr* fruits do not ripen, even when exposed to ethylene. Flowers do not senesce or abscise following fertilization nor do seedlings respond to ethylene, indicating that this mutation confers ethylene insensitivity throughout the plant. The mutation is the

consequence of a single amino acid change in the NR ethylene receptor. The lack of Nr ripening confirms the essentiality of ethylene perception for ripening.

In tomato, there are seven ethylene receptor genes (*LeETR1*, *LeETR2*, *NR*, *LeETR4*, *LeETR5*, *LeETR6*, and *LeETR7*). Five of these receptors have been shown to bind ethylene; two, LeETR6 and LeER7, were not tested [38] and references therein). Reduction of receptor content increases ethylene sensitivity, whereas increased receptor content has the opposite effect. **CTR1 family** members act downstream to the receptors. The single-copy *CTR1* gene was first identified in Arabidopsis. Loss of- function mutants exhibit a constitutive ethylene response, indicating that its function is to suppress the ethylene signaling pathway. Unlike *Arabidopsis*, there are four genes whose proteins exhibit significant homology to *CTR1*. Three of these genes (*tCTR1*, *tCTR3*, and *tCTR4*) can functionally complement the *Arabidopsis ctr1* mutant, indicating a degree of functional redundancy. Expression analysis suggests that *tCTR1* is the most highly expressed member of this family in the fruit and its mRNA abundance is induced during ripening and by ethylene [[38] and references therein]. **ETHYLENE-INSENSITIVE 2** (**EIN2**) acts genetically downstream of the ethylene receptors and CTR1 and encodes a protein with homology to the natural resistance-associated macrophage protein family of metal ion transporters [[39] and references therein]. EIN2 is localized within the ER, where it interacts with the kinase domain of each receptor in an ethylene-dependent manner. Phosphorylation of EIN2 by CTR1 retains EIN2 within the ER but ethylene dependent dephosphorylation leads to proteolytic cleavage of EIN2, resulting in translocation of the EIN2 C terminus into the nucleus, where it activates EIN3 and ethylene-dependent transcription [39]. Transcription factors that activate ethylene-responsive genes include the family of ***EIN3*-related genes**, *LeEIL1–4*. Antisense knock-downs of *LeEIL1*, *LeEIL2*, and *LeEIL3* significantly reduced ethylene sensitivity. All three of the genes are expressed throughout the plant, and each individual gene was capable of complementing an *Arabidopsis ein3* knockout. These results are consistent with these three genes having redundant function in tomato [[38] and references therein]. At the bottom of the signaling cascade is a family of transcription factors, the **ethylene response factors** (**ERFs**), that are members of the large AP2/ERF superfamily. Their expression is activated by the EIN3-like EILs.

Research on non-ripening tomato mutants has provided detailed insights into the regulation of climacteric ripening. One of the best studied of these tomato mutants is ripening-inhibitor (***RIN***), in which virtually all measured ripening phenomena, like the ethylene burst, change in color, fruit softening and the production of flavor compounds are inhibited . The dramatic phenotypic effect of the *rin* mutant suggests that RIN is a master regulator for

many ripening related processes. RIN/rin hybrids are widely used in commercial tomato production because of their extended shelf-life [[40] and references therein]. RIN is part of the MADS-box transcription factor family, known to function as DNA-binding protein dimmers consisting of two interacting MADS monomers [[40] and references therein]. RIN has been shown to directly interact with promoters of genes involved in the major pathways associated with ripening, like ethylene biosynthesis, ethylene perception, downstream ethylene response, cell wall metabolism, and carotenoid biosynthesis [41-43]. ***NON-Ripening*** (***NOR***) is another studied gene involved in regulation of tomato fruit ripening upstream to ethylene. It is a NAC transcription factor [44]. Complex interactions exist between RIN and NOR, with NOR acting upstream and downstream RIN [[44] and references therein].

The effect of applied ethylene concentrations on tomato flower drop

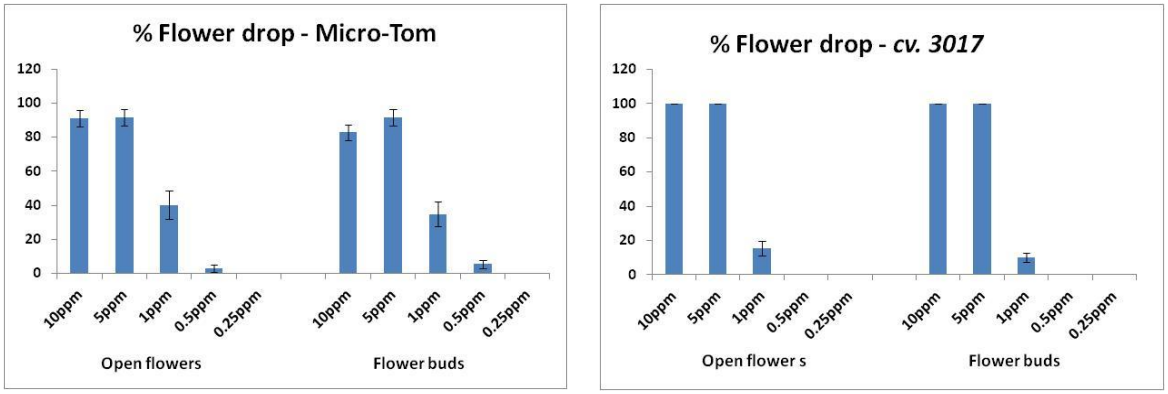


Figure 1. The effect of ethylene on % flower-drop in tomato genotypes '*MicroTom*' and *cv. Hazera 3017*' (*cv. 3017*). Results are the average of eight replicates ± SE. Flower drop was measures 1 hour after the treatment. Flower buds – at 4 to 1 days before opening.

The effect of ethephon (an ethylene-releaser) pre-treatment on pollen thermotolerance

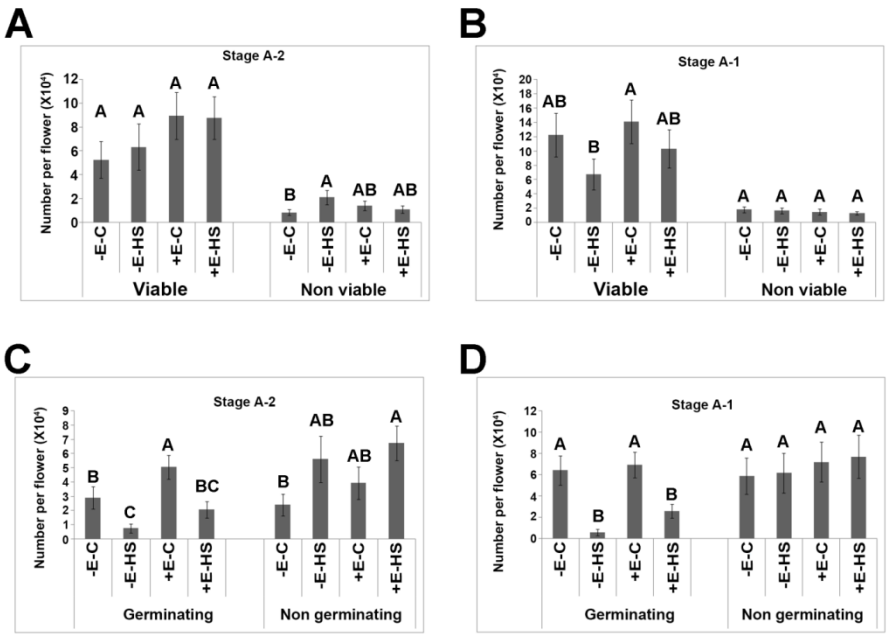


Figure 2. Effect of ethephon pretreatment of tomato Micro-Tom plants on pollen quality following exposure of the plants to short-term heat stress (HS). Plants were either pre-treated with ethephon (P-E-C, P-E-HS; soaked in a solution of 1 ppm for 18 h before applying either control or HS conditions) or not pretreated (P-C, P-HS; soaked in water). HS conditions were 2 h exposure to 50 ^{o}C, applied at 2 (A, C) and 1 days (B, D) before flower opening (developmental stages A-2 and A-1, respectively). Mature pollen grains were collected and pollen quality determined. Data are presented as mean values ± SE (n = 3 biological replicates) of number of Viable, Non viable (A, B), Germinating and Non germinating (C, D) pollen grains per flower (each replicate being an average of pollen derived from 8 flowers collected from different plants). C – control conditions; 2 h at 25 ^{o}C. In each pollen quality category (Viable, Non viable, Germinating, Non germinating), bars with different letters are significantly different by multiple comparison Tukey's HSD test (α = 0.05).

Use of tomato ethylene signaling mutants for testing the effect of HS on fruit number per plant and fruit weight

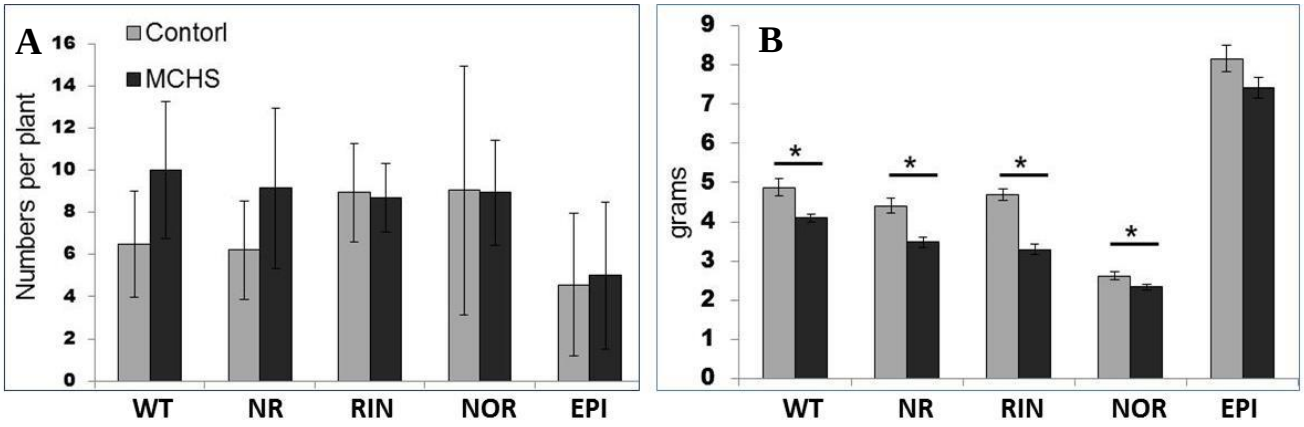


Figure 3. The effect of HS on fruit number per plant (A) and average fruit weight (B) in WT and tomato ethylene signaling mutant plants. Tomato (Micro-Tom) plants were grown at either control 26/23 ± 2 ^{o}C or mild chronic heat-stress conditions (MCHS) 35/23 ± 2 ^{o}C day/night temperatures. Results are average of two seasons. In each season, fruits were collected from 10 plants. Data are presented as mean values ± SE (n = 3 biological replicates). (*) - represent significantly different values by student t test analysis (p ≤ 0.05).

Use of tomato ethylene signaling mutants for testing the effect of HS on seed weight

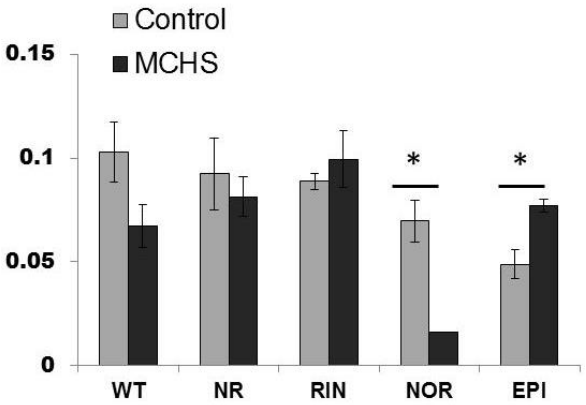


Figure 4. The effect of mild chronic heat-stress conditions (MCHS) on average seed weight (grams) in WT and ethylene signaling plants. Tomato (Micro-Tom) plants were grown at either control 26/23 ± 2 ^{o}C or MCHS (35/23 ± 2 ^{o}C day/night temperatures). Seeds were collected during two seasons and 30 seeds were weighed per replicate per treatment. Data are presented as mean values ± SE (n = 3 biological replicates). (*) - represent significantly different values by student t test analysis (p ≤ 0.05).

Do pollen grains have the capacity for ethylene production?

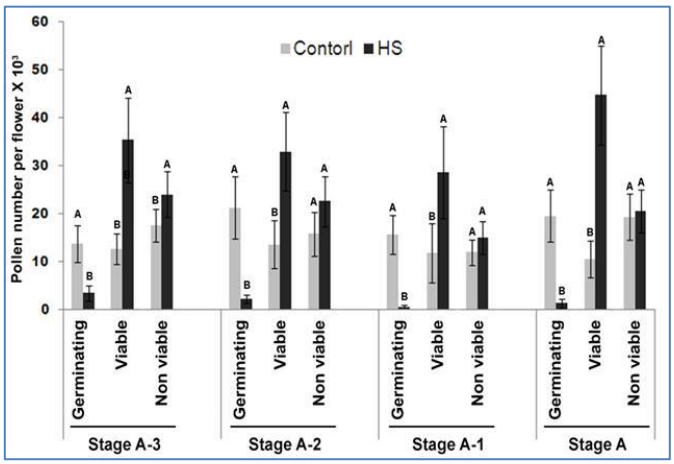
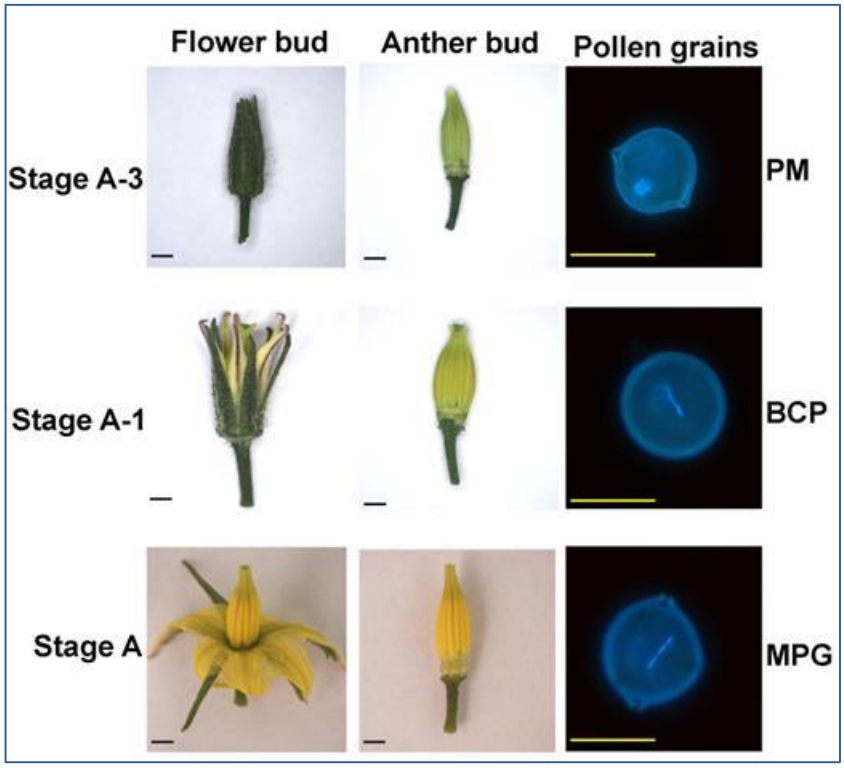


Figure 5. The effect of heat-stress (2 h 45 ^{o}C) on tomato pollen quality. Pollen quality was evaluated as described in [11], using tomato *cv. 3017* detached flowers, and the number of germinating, viable and non-viable pollen grains was recorded 3, 2, 1, 0 days after HS exposure (pollen grains experiencing HS at developmental stages A-3, A-2, A-1 and A, respectively). The flowers were maintained inside a 96 well plate until pollen quality analyses. The system was calibrated so that pollen quality was similar to quality of greenhouse-grown flowers (data not shown). Control - 2 h 25 ^{o}C. In each category, bars with different letters are significantly different by multiple comparison Tukey's HSD test (α = 0.05).



Picture 1. Flower, anther and pollen developmental stages used for ethylene production analyses.
PM – polarized microspores
BCP – bicellular pollen grains.
MPG – mature pollen grains.
Scale bar represents 4 mm for flower and anther buds and 200 µm for pollen grains. Pollen grains were stained with DAPI.
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Ethylene biosynthesis-, signaling- and responsive-genes expression

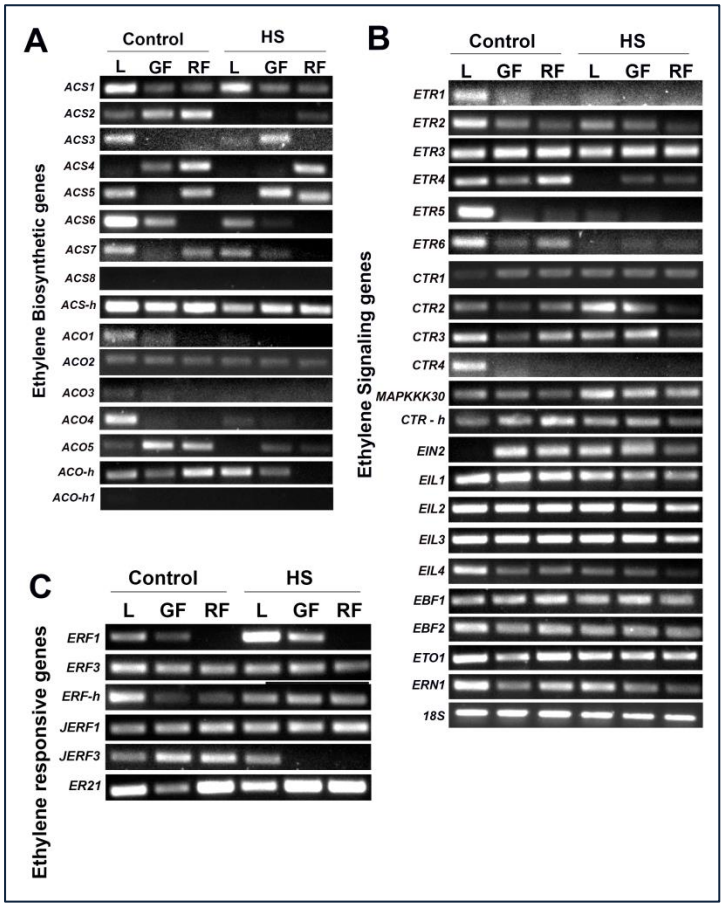

Figure 6: Expression levels of ethylene signaling genes in tomato leaf, mature green fruit and red fruit tissues at 'control' and heat-stress conditions. **A**. Ethylene biosynthesis genes. **B**. Ethylene signaling genes. **C**. Ethylene responsive genes. Expression levels were measured in leaves (L), green fruit (GF) and red fruit (RF) of tomato using semi-quantitative RT-PCR. HS - 2 hours exposure to 45 ^{o}C. Control - 2 hours exposure of fruits (green fruit and red fruit), branches with leaves to 25 ^{o}C. For all genes, the amplicon sequences were verified by sequencing. **ACS-h: solyc03g007070.**

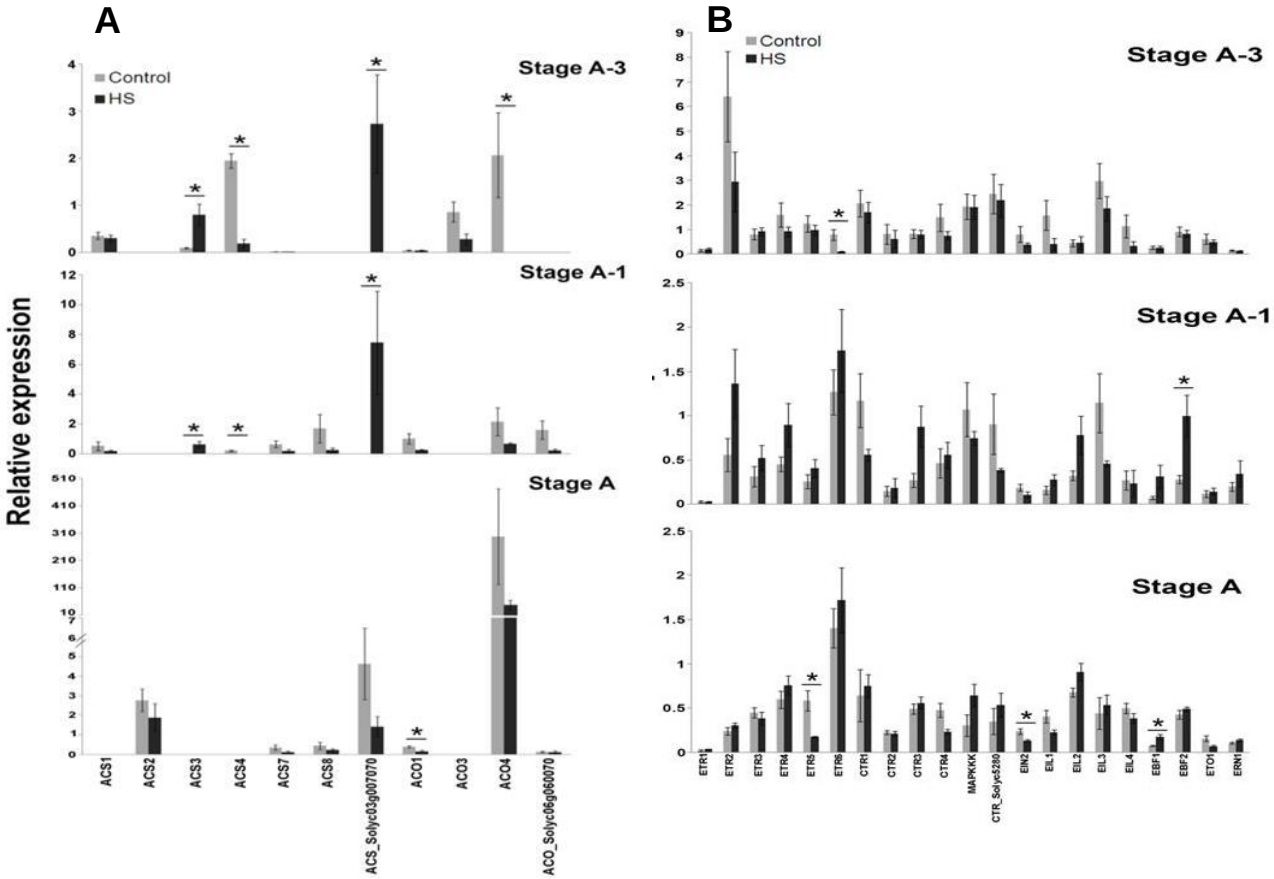

Figure 7. Expression levels of ethylene biosynthesis genes (*ACS* and *ACOs*) in tomato pollen grains (A) and anther walls (B) at control and heat-stress conditions. Tomato pollen and anther walls were collected from flowers of *cv. 3017* that were exposed to either control (2h 25 ^{o}C) or HS (2h 45 ^{o}C) conditions at developmental stages of 3, 1 and 0 days before flower opening (A-3, A-1 and A, respectively). Expression levels were measured using at least 3 biological replicates per sample. For each biological replicate, pollen and anthers were derived from at least 40 and 10 flower buds, respectively. Gene expression values are represented relative to the expression levels of 18S rRNA in the same cDNA sample. Expression data are the means (± SE). ** - represents significant difference at p-value <0.01 and * represent significant difference at p-value (<0.05).

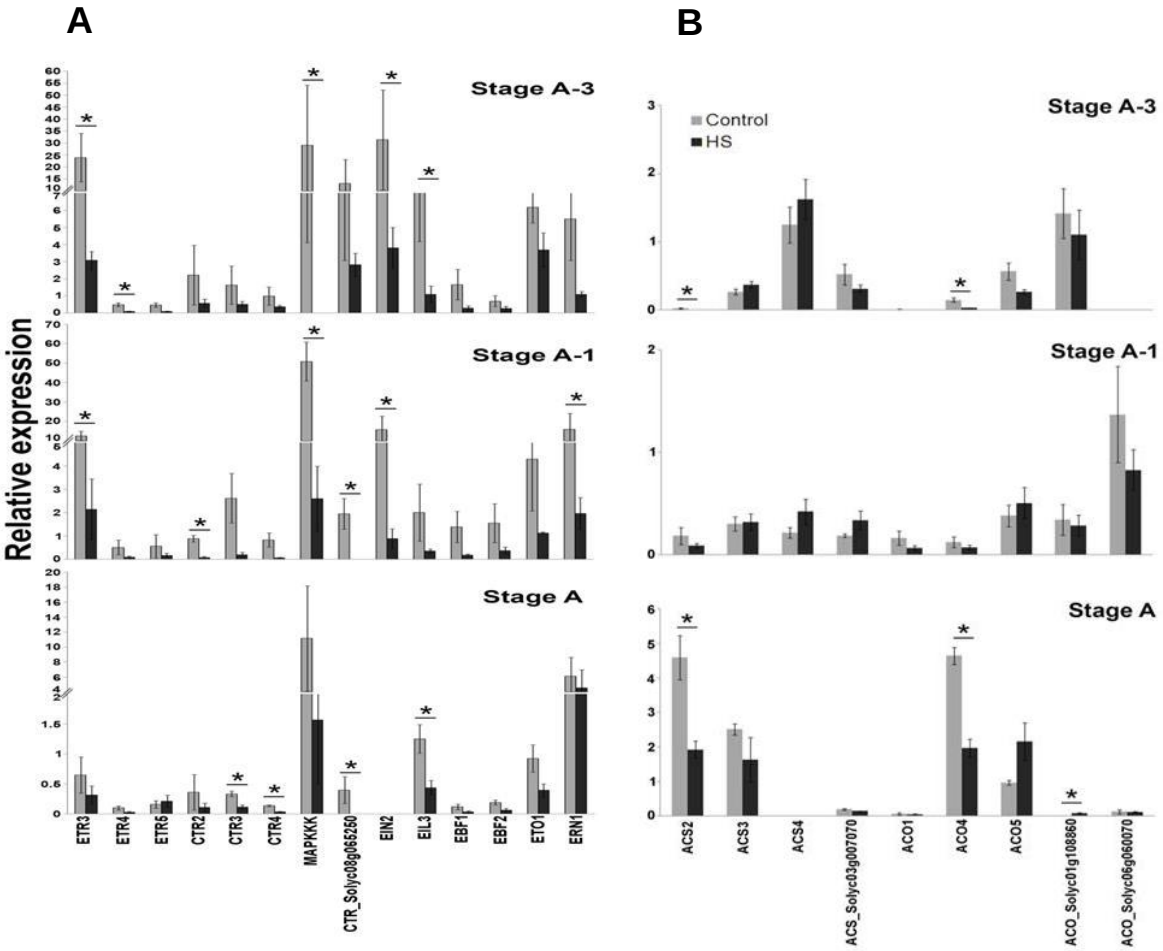

Figure 8. Expression levels of ethylene signaling genes in tomato pollen grains (A) and anther walls (B) at control and heat-stress conditions. Tomato pollen and anther walls were collected from flowers of *cv. 3017* that were exposed to either control (2h 25 ^{o}C) or HS (2h 45 ^{o}C) conditions at developmental stages of 3, 1 and 0 days before flower opening (A-3, A-1 and A, respectively). Expression levels were measured using at least 3 biological replicates per sample. For each biological replicate, pollen and anthers were derived from at least 40 and 10 flower buds, respectively. Gene expression values are represented relative to the expression levels of 18S rRNA in the same cDNA sample. Expression data are the means (± SE). ** - represents significant difference at p-value <0.01 and * represent significant difference at p-value (<0.05). For details of signaling genes see Appendix A scheme 1.

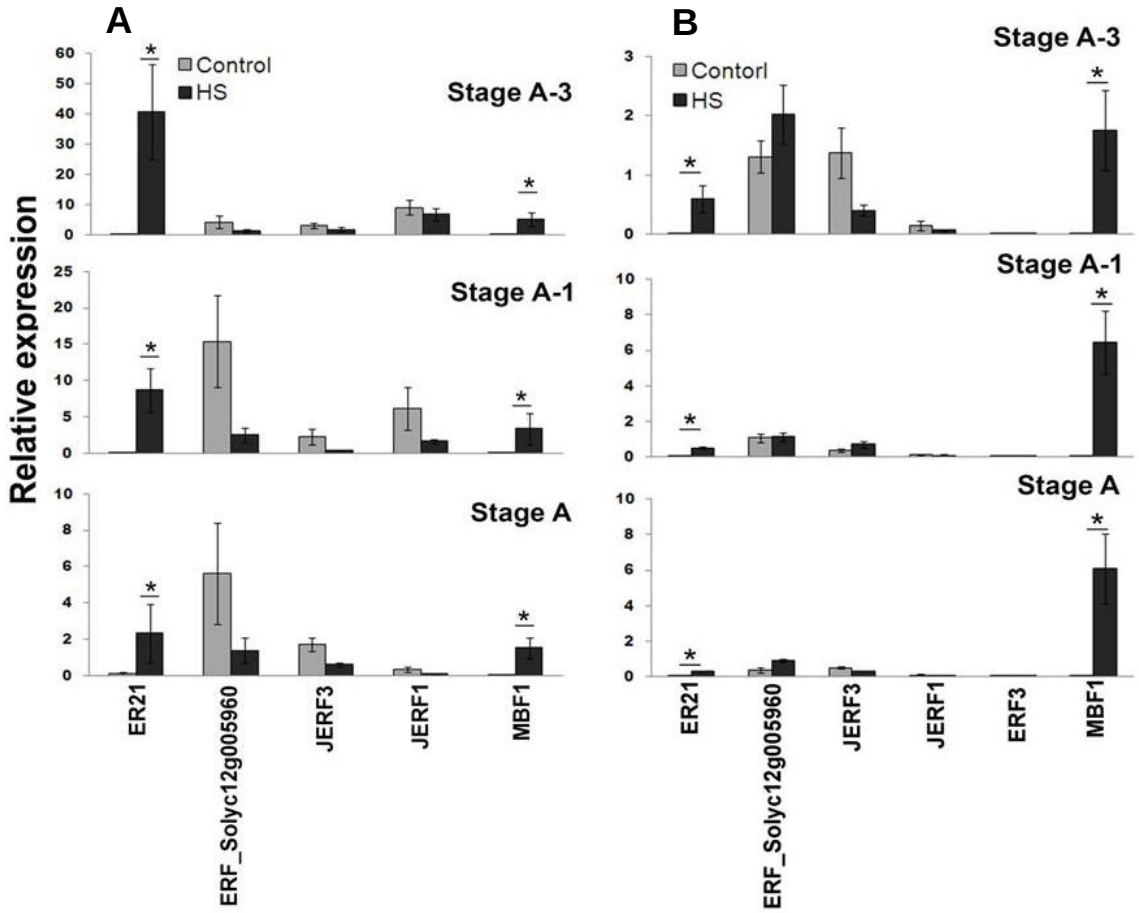

Figure 9. Expression levels of ethylene responsive genes in tomato pollen grains (A) and anther walls (B) at control and heat-stress conditions. Tomato pollen and anther walls were collected from flowers of *cv. 3017* that were exposed to either control (2h 25 ^{o}C) or HS (2h 45 ^{o}C) conditions at developmental stages of 3, 1 and 0 days before flower opening (A-3, A-1 and A, respectively). Expression levels were measured using at least 3 biological replicates per sample. For each biological replicate, pollen and anthers were derived from at least 40 and 10 flower buds, respectively. Gene expression values are represented relative to the expression levels of 18S rRNA in the same cDNA sample. Expression data are the means (± SE). ** - represents significant difference at p-value <0.01 and * represent significant difference at p-value (<0.05).

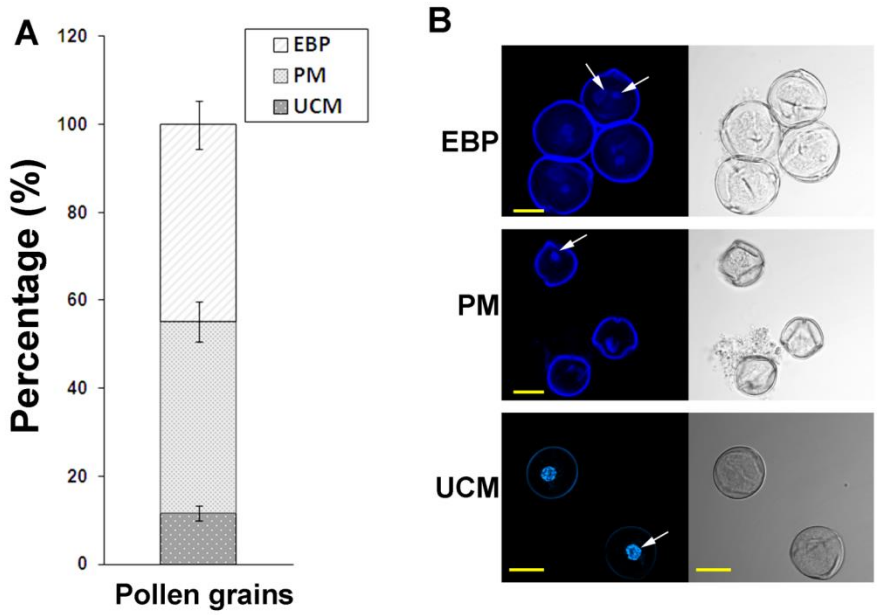

Figure 10. Percentage of individual pollen stages in the samples (combined A-4 & A-3 stages) used for proteome analysis. Unicellular – UCM; Polarized microspores - PM; early bicellular – EBP. Scale bar represents 10 µm. Pollen grains were stained with DAPI.

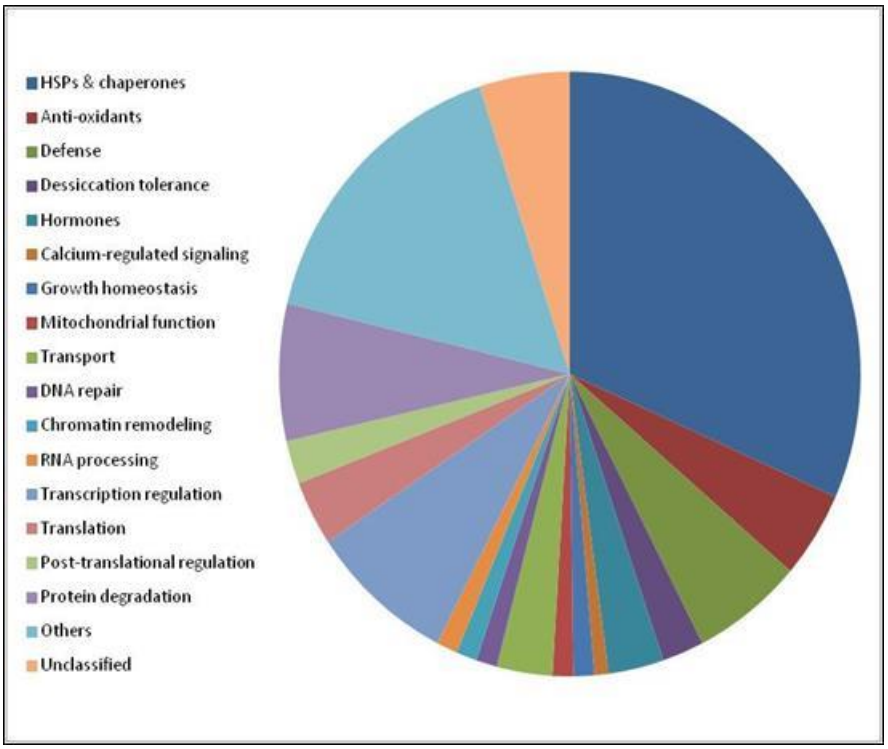

Figure 11. Percentage of transcripts belonging to a given function, calculated for the differentially expressed (≥2.5-fold) genes in the enriched GO category 'response to stress'.

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מטרות המחקר תוך התייחסות לתוכנית העבודה.

מטרות המחקר בהתאם לתוכנית העבודה: 1. אפיון תפקיד מסלול האתילן בתגובה- ובעמידות-לחום של גרגרי אבקה של עגבנייה. 2. אפיון יחסי הגומלין בין מסלול האתילן ומסלולים אחרים בתגובה- ובעמידות-לחום של גרגרי האבקה.

אילו ממטרות המחקר הושגו בעבודת המחקר הנוכחית

כל המטרות הושגו.

עיקרי התוצאות.

לשם ביסוס מעורבות אתילן בתפקוד גרגרי אבקה בתנאי עקת-חום: א. בחנו ההשפעה של טיפול בצמחים/בתפרחות בחומר המשחרר אתילן ('אתפון'). מצאנו שטיפול באתפון לפני חשיפת הצמחים לעקת-חום גרם לעלייה משמעותית באיכות האבקה. ב. השתמשנו במוטנטים של עגבנייה הפגומים במסלול האתילן, כולל מוטנט ברצפטור Nr, המוטנטים non-ripening (nor) ו- ripening inhibitor (rin) ומוטנט epinasty (epi) המייצר אתילן ברמה מוגברת. נבחנה השפעת המוטציות על איכות האבקה, החנטה וכושר ייצור הזרעים בתנאי עקת-חום. נמצא שמוטנט *epi* מראה איכות אבקה גבוהה יותר, ייצור אחוז גבוה יותר של פירות עם זרעים, וייצור כמות גדולה יותר של זרעים לפרי. ממצא זה מחזק הממצאים של הטיפול המקדים באתפון, ומבסס את התפקיד המשמעותי שיש לאתילן בשמירה על איכות האבקה בעגבנייה בתנאי עקת-חום. מעניין גם לציין את התנהגות המוטנט *rin* המראה השפעה מיטיבה על איכות האבקה וייצור פירות עם זרעים בחום – בהשוואה ל-WT.

בנוסף, על מנת לבחון באם גרגרי אבקה מכילים מרכיבים של מערכת הביוסינתיזה, החישה, והולכת הסיגנל של אתילן, בחנו ביטוי מספר רב יחסית של גנים המרכיבים מערכות אלה בגרגרי אבקה מתפתחים כמו גם ברקמות המאבק התומכות את התפתחות האבקה. כביקורת חיובית נבחן ביטוי גנים אלה ברקמות עלים, פרי ירוק ופרי בשל. מצאנו שלגרגרי האבקה כושר ייצור אתילן כמו גם מערכת גנים לביוסינתיזה, חישה והולכת הסיגנל של אתילן. נמצא גם, לגבי חלק מהגנים, פרופיל ביטוי ייחודי לאבקה. ניתן לראות ביטוי דיפרנציאלי בשלבי התפתחות שונים של האבקה כמו גם הבדלים בדגם הביטוי בין רקמות המאבק לאבקה. לגבי חלק מהגנים ניתן לראות שינוי ברמות הביטוי באבקה בעקבות חשיפה לעקת-חום.

מעבר לכך, השתמשנו במערכת שביססנו במהלך מחקר זה לזיהוי מנגנונים המעורבים בתגובת גרגרי האבקה לחום, ומנגנונים המעורבים בעמידות לחום המתווכת ע"י אתילן, זאת ע"י שימוש בגישה פרוטיאומית. התוצאות הראו שעקת-חום פוגעת במנגנונים חיוניים להתפתחות האבקה, כולל הומאוסטאזיס של חלבונים. טיפול מקדים באתפון שינה את פרוטאום האבקה בתגובה לחום כך שהיה דומה לפרוטאום האבקה שהתקבל בתגובה לתנאי ביקורת, כולל עלייה בביטוי חלבונים המבקרים תרגום וחלבונים במעגל ה-TCA. מעבר לכך, טיפול באתפון גרם לעלייה במנגנוני הגנה המתמודדים עם נזקי חמצון. בנוסף, מצאנו שלגרגרי האבקה היכולת לעמידות נרכשת לחום, וננקטה גישה טרנסקריפטומית על מנת לזהות המנגנונים המעורבים.

מסקנות מדעיות וההשלכות לגבי יישום המחקר והמשכו. האם הושגו מטרות המחקר לתקופת הדוח?

המטרות לתקופת הדו"ח הושגו. בוססה מעורבות אתילן בעמידות גרגרי אבקה של עגבנייה לחום, וזוהו מנגנוני עמידות-לחום המופעלים ע"י טיפול באתפון. נמצאה חפיפה בין מנגנונים אלה למנגנונים המעורבים בעמידות נרכשת לחום, כולל מעורבות חלבוני עקת-חום, אנטיאוקסידאנטים, ופקטורים המעורבים בתרגום.

בעיות שנותרו לפתרון ו/או שינויים (טכנולוגיים ,שיווקיים ואחרים) שחלו במהלך העבודה; התייחסות המשך המחקר

התוצאות שהתקבלו עשויות לשמש להעלאת איכות האבקה בעגבנייה וייצור פירות עם זרעים בתנאי עקת-חום. התקבלו מאגרי מידע ברמה הפרוטאומית והטרנסקריפטומית לשימוש לשם אפיון גנים ושימוש בהם כסמנים לעמידות לחום במחקר המשך.

הפצת הידע שנוצר בתקופת הדו"ח: **פרסומים בכתב** -<u>ציטט</u> ביבליוגרפי כמקובל בפרסום מאמר מדעי;
פטנטים -יש לציין שם ומס 'פטנט ;**הרצאות וימי עיון** -יש לפרט מקום ,תאריך ,ציטוט ביבליוגרפי של התקציר כמקובל בפרסום מאמר מדעי.

פרסום הדוח: אני ממליץ לפרסם את הדוח: (סמן אחת מהאופציות)

ללא הגבלה - (בספריות ובאינטרנט)

✓ **<u>בשלב זה חסוי</u>** – לא לפרסם. אנו במהלך סכום התוצאות למאמרים.

האם בכוונתך להגיש תוכנית המשך בתום תקופת המחקר הנוכחי ? כן