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You are cordially invited to attend the public defense of my doctoral thesis, entitled: "Genetics and Physiology of Pollen Thermotolerance in Tomato" on November 23, 2016 at 16:30 h in the Aula of Radboud University, Comeniuslaan 2, Nijmegen, Netherlands.

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Genetics and Physiology of Pollen
Thermotolerance in Tomato

Jiemeng Xu
Genetics and Physiology of Pollen
Thermotolerance in Tomato

Proefschrift
ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.H.J.M. van Krieken,
volgens besluit van het college van decanen
in het openbaar te verdedigen op woensdag 23 november 2016
om 16.30 uur precies

door

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Genetics and Physiology of Pollen
Thermotolerance in Tomato

Doctoral Thesis

to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. J.H.J.M. van Krieken,
according to the decision of the Council of Deans
to be defended in public on Wednesday, November 23, 2016
at 16.30 hours

by

Jiemeng Xu
Born on October 22, 1987
in Hunan (China)
To my grandma!
致我的奶奶！
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<th>Definition</th>
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<tbody>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>PMC</td>
<td>Pollen mother cell</td>
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<tr>
<td>MH</td>
<td>Mild heat</td>
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<tr>
<td>CWIN</td>
<td>Cell wall invertase</td>
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<td>HSP</td>
<td>Heat shock protein</td>
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<tr>
<td>HSF</td>
<td>Heat stress transcription factor</td>
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<tr>
<td>HSR</td>
<td>Heat stress response</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>QTL</td>
<td>Quantitative trait locus</td>
</tr>
<tr>
<td>GRX</td>
<td>Glutaredoxin</td>
</tr>
<tr>
<td>DBA</td>
<td>Day(s) before anthesis</td>
</tr>
<tr>
<td>GSEA</td>
<td>Gene set enrichment analysis</td>
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<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
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<tr>
<td>FDR</td>
<td>False discovery rate</td>
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<tr>
<td>DEG</td>
<td>Differentially expressed gene</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>APX</td>
<td>Ascorbate peroxidase</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>CSD</td>
<td>Copper-zinc superoxide dismutase</td>
</tr>
<tr>
<td>MSD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>GR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>GPX</td>
<td>Glutathione peroxidase</td>
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<tr>
<td>LSD</td>
<td>Least significant difference</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>CMH</td>
<td>Continuous mild heat</td>
</tr>
<tr>
<td>RH</td>
<td>Relative humidity</td>
</tr>
<tr>
<td>PV</td>
<td>Pollen viability</td>
</tr>
<tr>
<td>PN</td>
<td>Pollen number</td>
</tr>
<tr>
<td>IN</td>
<td>Inflorescence number</td>
</tr>
<tr>
<td>FPI</td>
<td>Flowers per inflorescence</td>
</tr>
<tr>
<td>FS</td>
<td>Fruit set</td>
</tr>
<tr>
<td>FF</td>
<td>Female fertility</td>
</tr>
<tr>
<td>SR</td>
<td>Seedling survival rate</td>
</tr>
<tr>
<td>IL</td>
<td>Ion leakage</td>
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<tr>
<td>TGRC</td>
<td>Tomato genetics resource center</td>
</tr>
<tr>
<td>AL</td>
<td>Anther length</td>
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<tr>
<td>SL</td>
<td>Style length</td>
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<tr>
<td>SP</td>
<td>Style protrusion</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>KASP</td>
<td>Kompetitive allele specific PCR</td>
</tr>
<tr>
<td>SIM</td>
<td>Standard interval mapping</td>
</tr>
<tr>
<td>CIM</td>
<td>Composite interval mapping</td>
</tr>
<tr>
<td>LOD</td>
<td>Logarithm of the odds ratio</td>
</tr>
<tr>
<td>ZFN</td>
<td>Zinc Finger Nuclease</td>
</tr>
<tr>
<td>TALEN</td>
<td>Transcription Activator-Like Effector Nuclease</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered Regularly Interspaced Short Palindromic Repeat</td>
</tr>
<tr>
<td>CRISPR/Cas</td>
<td>CRISPR associated proteins</td>
</tr>
<tr>
<td>GMO</td>
<td>Genetically Modified Organism</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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CHAPTER 1

General introduction

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Global warming and crop production

Over the period from 1880 to 2012, the earth’s surface temperature showed a 0.65-1.06 °C increase and temperature projections point to continued warming in the near future (Pachauri et al., 2014). This so-called process of “global warming” has been receiving increasing attention in recent years, due to its far-reaching effects on human society. This includes changes to overall climate and weather (“climate change”), features like the sea level and ecosystem functioning, and in the end our economy and vital aspects, like fresh water availability and food security. The two main policies to address climate change are climate change mitigation (e.g. reduction of greenhouse gas emissions or capturing of carbon dioxide through geoengineering) and adaptation to the impacts of climate change. Given the unprecedented rate at which global warming occurs currently, it seems reasonable to invest substantial efforts in both directions.

A major way in which high temperature impacts food security is through adverse effects on crop productivity (Wheeler and von Braun, 2013). Moderate increase in temperature and extension of the growing season may, in fact, be beneficial for crop production, but global warming comes with considerable regional and seasonal deviations to the average yearly increase in temperature, as well as an increase in the frequency and severity of extreme local temperature fluctuations (Pachauri et al., 2014). In particular, hot summers and heat waves reduce agricultural yields. For instance, maize and soybean production was negatively correlated with the maximum temperatures recorded during the summer in North America (Mishra and Cherkauer, 2010) and worldwide yield reductions were expected for wheat, rice and maize in both tropical and temperate regions under a scenario of 2 °C of warming without adaptation (Challinor et al., 2014). Even in more moderate climates, such as in Europe, it was predicted that the limiting factor for wheat production will be heat, rather than drought in the future (Semenov and Shewry, 2011). Thus, as part of efforts to adapt human society to the new, warmer climate, research to facilitate the development of more heat-tolerant crops is urgently necessary.

Plants and high temperature

As sessile organisms, plants are inevitably exposed to their environmental temperature throughout their life cycle and may even overheat during the day due to radiation of the sun. Plants have the ability to cool somewhat by transpiration, but to a limited extent, only. The effect of high temperature on plants depends on the absolute temperature, the rate of temperature increase and the length of exposure. Furthermore, heat susceptibility differs among developmental stages and depends on the physiological state of the plant. The latter can be seen for example from interaction between high temperature and water
availability (Barnabás et al., 2008), as well as from the phenomenon of heat acclimation, where the history of a plant's growth conditions influences its heat tolerance in the short or longer term (Vierling, 1991; Wang et al., 2011). High temperature directly affects the structure of biomolecules such as DNA, RNA, proteins and lipids and thereby changes fundamental metabolic processes like photosynthesis, respiration and redox homeostasis (Mittler et al., 2012). At organismal level, this leads to disturbance of growth and development (Bokszczanin et al., 2013). Harmful effects manifest themselves especially after the transition to reproductive phase and may cause heat sterility. This can be due to defective gamete development, unsuccessful fertilisation and/or interrupted early embryo development (Bagha, 2014; Hedhly et al., 2009; Zinn et al., 2010). Generally, developing pollen and ovules experience high temperature simultaneously, but the former are more sensitive to heat (Peet et al., 1998; Dupuis and Dumas, 1990). Taken together, the generative stage is more sensitive to high temperature, and the impaired pollen fertility is one of the main reasons for heat-induced yield loss.

**Pollen development and high temperature**

Pollen formation inside the anther locules initiates from diploid pollen mother cells (PMCs) that undergo meiosis to give rise to four haploid daughter cells as a tetrad enclosed by callose. After release, the free microspores develop, enlarge and divide asymmetrically (pollen mitosis I) into a large vegetative and a small generative cell, the latter of which is engulfed into the cytoplasm of the former (Figure 1). The fates of these two types of cell are highly divergent: the generative cell undergoes a second mitosis (pollen mitosis II), either during pollen development or during pollen tube growth, to form two sperm cells, while the vegetative cell provides a stable environment for the generative/sperm cell development and delivers the sperm cells to the female gamete via the formation of a pollen tube (Brukhin et al., 2003; McCormick, 2004). During differentiation of the PMCs, the most inner anther wall layer forms tapetum (Borg et al., 2009; Goldberg et al., 1993; Ma, 2005). This tissue is metabolically active especially at very early microspore stage and provides the developing microspores with carbohydrates, nutrients, enzymes and wall compounds (McCormick, 2004; Pacini et al., 1985; Scott et al., 2004). The tapetum development is highly coordinated with pollen development; it starts to degenerate already shortly after the release of the microspores and in some species is fully degraded before pollen mitosis I (Wu and Cheung, 2000). The correct functioning of the tapetum, but also its timely degradation are essential for pollen development (Li et al., 2006; Parish and Li, 2010; Yi et al., 2016).

Pollen development has been described to be sensitive to high temperature in many
species, both to short severe heat shock and long-term mildly elevated temperature (Harsant et al., 2013; Pressman et al., 2002; Saini et al., 1984; Sakata et al., 2000; Young et al., 2004). Because pollen development consists of such distinct processes, the timing of exposure to high temperature plays a crucial role in determining the effect of heat on pollen.

Figure 1. Schematic illustration of tomato pollen development.

Microsporocyte (also called PMC) which is harboured in loculus of anther gives rise to the formation of tetrad through meiosis. Afterwards, free microspores are released, polarized and divided into binucleate pollen with the generative cell engulfed in the vegetative cell. Further development of binucleate pollen forms mature pollen.

**Heat stress affects male meiosis**

Meiosis, a unique type of cell division, gives rise to daughter cells with halved the DNA content of somatic cells and produces novel allele combinations by chromatic segment exchange (Wijnker and Schnittger, 2013). In barley, mild heat (MH) led to premature initiation of male meiotic prophase I (Oshino et al., 2007). Also, high temperature was associated with increased crossing-over during male meiosis (Francis et al., 2007; Powell and Nilan, 1963). Coincidence of severe heat with meiosis also causes more severe disorder of meiotic behaviour, like chromosome migration and cytokinesis. For instance in wheat, laggard chromosomes and micronuclei were observed, indicating unbalanced chromosome separation (Omidi et al., 2014; Rana, 1965; Rezaei et al., 2010). In rose, high temperature was shown to disrupt spindle orientation during meiosis II, resulting in dyads of diploid microspores (Pécrix et al., 2011). Also in tomato, dyads were observed after exposure to severe heat shock (Müller and Rieu, personal communication). Most likely, these aberrations are due to a direct physical effect on cytoskeleton dynamics (De Storme and Geelen, 2014).
Exposure to heat at the microspore stage affects the tapetum

Tomato flower buds at the meiosis to early microspore stage were found to be most sensitive to a short heat shock of 40°C (Iwahori and Takahashi, 1964; Iwahori, 1965), comparable to the cases of rice and Arabidopsis (Endo et al., 2009; Kim et al., 2001). Also, sensitivity of pollen development to long-term MH conditions was found to be high in the phase that encompasses the meiosis to microspore transition stage in tomato, as well as cowpea, wheat and barley (Ahmed et al., 1992; Saini and Aspinall, 1982; Sakata et al., 2000; Sato et al., 2002). A number of studies suggested that the hypersensitivity at this pollen developmental stage was, in fact, mediated by the tapetum (De Storme and Geelen, 2014; Parish et al., 2012). In rice, tapetal gene expression was affected by heat treatment (Endo et al., 2009). When exposed to heat stress, the tapetum cells were degenerated either prematurely, as shown in examples of wheat and barley (Oshino et al., 2007; Saini et al., 1984) or too late, as reported for tomato (Iwahori, 1965). A detailed cytological study of snap bean tapetum development upon high temperature showed that heat caused malformation of endoplasmic reticulum, probably affecting the secretory activity of the tissue (Suzuki et al., 2001).

Sugar metabolism in anthers is sensitive to heat

The important role of carbohydrate metabolism and transport in the response of plants to external stimuli has been widely documented (Eveland and Jackson, 2012; Keunen et al., 2013; Ruan et al., 2010; Wingler and Roitsch, 2008). Being a heterotrophic organ, anther, including the developing pollen, constitute metabolically active sink. Pollen formation depends on the tight control of sugar content: in the course of pollen development, the content of soluble sugar and starch is maintained stably at low level during early stages until the first mitosis, after which the starch accumulates to a peak concentration in binucleate microspores and then gradually degrades, leading to the high accumulation of soluble carbohydrates at anthesis stage (Pacini et al., 2006; Pressman et al., 2012). Carbohydrates not only provide energy to drive developmental processes, but also function as osmolytes (Paupière et al., 2014). In agreement with the important role of carbohydrates, silencing genes involved in carbohydrate metabolism and allocation, such as the invertases NIN88 in tobacco and LIN5 in tomato, led to the loss of functional pollen (Goetz et al., 2001; Zanor et al., 2009).

Sucrose is the main form of photosynthetic assimilate exported from source tissue. Its unloading and uptake in symplastically isolated cells, like the microspores, predominantly depends on the activity of cell wall invertase (CWIN) (Jain et al., 2007; Paupière et al., 2014). Indeed, CWIN activity was maintained at high level in developing pollen (Pressman et al., 2012). Heat stress lowered down the expression of CWIN genes in microspores.
and anthers (Jain et al., 2007; Sato et al., 2006) and resulted in reduced sugar uptake and starch accumulation in tomato microspores (Firon et al., 2006; Pressman et al., 2002), accompanying by ectopic accumulation of the carbohydrates in surrounding anther compartments, such as the locular fluid (Pressman et al., 2002; Sato et al., 2006). Likewise, starch deposition in binucleate microspores was reduced in heat stressed sorghum plants (Jain et al., 2007). In line with the association, high temperature had less adverse effects on starch accumulation in heat-tolerant tomato plants (Firon et al., 2006).

A putative role for unfolded proteins in heat-induced pollen failure
In contrast to the direct effects of heat on cytoskeleton stability to explain the meiotic anomalies (De Storme and Geelen, 2014), no strong hypotheses have been generated regarding the primary mechanism by which heat disrupts pollen development. Given the general observation that heat results in structural instability of proteins, this might be involved in pollen failure.

Heat shock protein (HSP), as shown by its name, was firstly reported to be induced in response to heat stress (Ritossa, 1962; Tissiéres et al., 1974). The HSPs are categorised into five groups according to their molecular weight, namely HSP100, HSP90, HSP70, HSP60, and small HSPs. A number of members are found in each family, which indicates the functional diversity of HSPs (Al-Whaibi, 2011; Wang et al., 2004). Members from the different HSP families work collaboratively to rescue the correct conformation of proteins that are denatured by stressful conditions (Al-Whaibi, 2011; Vierling, 1991; Wang et al., 2004). The expression of HSP is transcriptionally regulated by heat stress transcription factors (HSFs), which also exhibit a high diversity in terms of structure and function in planta (Baniwal et al., 2004; Scharf et al., 2012; von Koskull-Döring et al., 2007). The HSFs and HSPs work together in cascades to modulate heat stress response (HSR) and heat tolerance. For instance, HSFA2, Hsa32 and HSP101 worked together to regulate acquired thermotolerance in rice and Arabidopsis (Charg et al., 2006; Lin et al., 2014; Wu et al., 2013). In tomato, interactions between HSFA1, HSFA2, HSP70, HSP90 and HSP17 were proposed to modulate the HSR at different phases of heat stress (Hahn et al., 2011; Scharf et al., 2012). As for reproductive tissue, however, several studies showed that classical sets of the HSPs were hardly induced in mature or germinating pollen upon heat shock (Hopf et al., 1992; Frova et al., 1989; Cooper et al., 1984; Dupuis and Dumas, 1990). Some HSFs and HSPs could be induced by heat stress at earlier stages of pollen development (in microspores), but the level of induction was less than in vegetative tissue, and decreased at later stages (Fragkostefanakis et al., 2016; Gagliardi et al., 1995; Frova et al., 1989; Volkov et al., 2005). This may lead to the hypothesis that the heat sensitivity of developing pollen is due to a relatively weak HSR (Müller and Rieu, 2016). In line with this, two heat-tolerant
tomato accessions were shown to contain constitutively higher expression of several HSFs and HSPs (Bita et al., 2011; Frank et al., 2009). Also, ectopic expression of Arabidopsis HSP101 in tobacco improved pollen germination ability after heat challenge (Burke and Chen, 2015) and over-expression of HSF A2 in tomato tapetum enhanced HSPs expression and gave rise to better thermotolerance of developing pollen (Li, 2015).

Reactive oxygen species and heat-induced pollen defects

Similar to unfolded proteins, accumulation of reactive oxygen species (ROS) seems to be a general response to high temperature exposure. ROS are formed as by-products in various aerobic metabolic pathways within different cellular compartments, like chloroplasts, mitochondria, and peroxisomes and also within apoplast region through NADPH oxidases (Apel and Hirt, 2004; Das et al., 2015; Jajic et al., 2015; Mittler et al., 2004; Rhoads et al., 2006). Under steady state conditions, the content of ROS is tightly controlled by their production and removal to ensure proper cell functioning. Taking anther and pollen development as an example, Arabidopsis and rice mutants with less ROS produced at certain developmental stage exhibited retarded tapetum degradation and therefore loss of pollen fertility (Xie et al., 2014; Yi et al., 2016). On the other hand, excessive ROS in rice mads3 mutant at a later stage of microgametogenesis led to pollen abortion (Hu et al., 2011). Higher ROS level was found in rice male sterile lines than that in fertile lines at meiotic and early microspore stage (Luo et al., 2013; Wan et al., 2007). These data confirm that ROS level and timing must be finely regulated to ensure the production of viable pollen. However, the equilibrium between ROS production and removal can be perturbed by abiotic stress factors (Apel and Hirt, 2004). A number of studies reported that, upon heat stress, plant cells accumulate excessive amount of ROS (Dat et al., 1998; Morgan et al., 1986; Vacca et al., 2004; Volkov et al., 2006; Wu et al., 2012), forming a secondary oxidative stress, which damages membranes, proteins, lipids and DNA (Mittler, 2002; Sharma et al., 2012). The involvement of ROS in heat-induced pollen abortion is not well established. Treatment with antioxidants before heat exposure seemed to improve rice and wheat pollen quality (Fahad et al., 2016; Kumar et al., 2014). Moreover, the transcripts of some ROS scavenger genes, which are normally strongly induced by ROS, were up-regulated by heat in anthers or microspores (Bita et al., 2011; Frank et al., 2009; Li, 2015). Thus, together, it seems valid to speculate that ROS is relevant in pollen defects under heat.
**General introduction**

**Natural variation in pollen thermotolerance**

Driven by mutation and influenced by natural selection and the level of gene flow, species may accumulate considerable genetic diversity. This means that in a given environment, different individuals from the same species may vary in phenotype. As an alternative approach to study a biological process in a specific genotype, much can also be learned from correlating characteristics among genotypes. Surveys of sets of genotypes of various species revealed considerable phenotypic variations in terms of reproductive success under high temperature conditions (Bac-Molenaar et al., 2015; Opeña et al., 1992; Rainey and Griffiths, 2005; Warrag and Hall, 1983). In some cases, better reproductive success was shown to be associated with enhanced pollen quality under high temperature (Dane et al., 1991). As described before, the natural variation was also used to associate pollen thermotolerance with \( HSF \) and \( HSP \) expression (Bita et al., 2011; Frank et al., 2009), and sugar content (Firon et al., 2006).

Phenotypes may also be correlated to genome composition, thereby enabling analysis of the genetic architecture of a phenotypic trait. This knowledge, in turn, may support the unravelling of underlying molecular physiological mechanisms. A very limited number of quantitative genetic loci (QTLs) affecting reproductive success under heat have so far been identified, in *Arabidopsis*, cowpea, rice, wild rice and tomato (Bac-Molenaar et al., 2015; Grilli et al., 2007; Li et al., 2015; Lin et al., 2010; Lucas et al., 2013; Xiao et al., 2011; Ye et al., 2012). However, only in case of rice, the effect was attributed to pollen viability as a subcomponent: two QTLs accounted for 9-15% phenotypic variation in pollen fertility under heat stress (Xiao et al., 2011).

**Scope of the thesis**

Taken together, a lot of knowledge has been gained on the impact of high temperature on pollen development. However, a clear understanding of the primary effect of heat on pollen and the reason for the exceptional heat sensitivity of pollen is lacking. The studies presented in this thesis are an effort to better describe the initial deviations in pollen development imposed by MH treatment, to test the involvement of ROS and to identify genetic determinants of heat tolerance.

Chapter 2 is a descriptive study of pollen development after a minimal effective period of MH at the cytological and transcriptomic level. The main conclusion is that 4 days of MH containing the meiosis to early microspore stage affects sugar metabolism and leads to pollen failure after pollen mitosis I.

Chapter 3 assesses the role of ROS in pollen failure under MH, by looking at ROS damage, ROS-related gene expression and enzyme activity, and the phenotype of tomato lines with enhanced antioxidant ability by melatonin application or by ectopic expression of
Arabidopsis glutaredoxin (GRX) gene, which maintains redox status by reducing oxidised proteins. A definitive conclusion on the role of ROS could not be drawn.

Chapter 4 describes the natural diversity of thermotolerance among 13 tomato cultivars and a correlation analysis among measured traits. The main conclusions are that there is a considerable variation for reproductive trait performance under MH and that pollen viability limits seeded fruit set under this condition.

Chapter 5 reports a QTL analysis for the performance of reproductive traits under MH in a F$_2$ population derived from two phenotypically contrasting cultivars. Highly significant QTLs are identified for pollen viability, pollen number, style length, anther length and stigma position. In addition, QTLs for inflorescence production and the number of flowers per inflorescence are found as well. Main conclusions are that thermotolerance of pollen viability and pollen number are genetically distinct traits and that stigma position is mainly determined by style length.

Chapter 6 is a synthesis of obtained results and discussion of opportunities for application in the context of breeding and crop management.
References

Chapter 1


Mild heat during early flower development results in pollen abortion at late stages

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Chapter 2

Abstract

The negative effect of heat on plant reproductive success has been widely reported and has been ascribed in part to the heat sensitivity of pollen development. Here, the effect of mild heat (MH) on tomato pollen was characterised. Pollen development was most sensitive to MH at 11-9 days before anthesis (DBA), and a 3- or 4-day period of MH around this period led to substantial pollen failure, whereas little effects were found from 1- or 2-day MH treatment. The sensitive time frame encompassed the stages of pollen mother cells, meiosis and early microspores. Cytological analysis of 4-day MH treated anthers (at 13-10 DBA) showed that developmental deviations were first detected around 7 days later (at 3 DBA), i.e. after pollen mitosis, and that 50% of MH treated pollen died between 3 and 1 DBA. Binucleate pollen from MH treated buds accumulated fewer and smaller starch granules. Microarray analysis of meiosis to early microspore stage anthers at the day after a non-damaging 1-day and damaging 4-day MH treatment revealed differential expression of cell wall invertase, i.e. up-regulation after 1-day MH, but normal or even reduced levels after 4-day MH. Thus, an early effect of MH on carbohydrate metabolism might result in problems with starch deposition and pollen viability during pollen maturation.
Introduction

Temperature projection points out that the global surface temperature will continue to rise, and that periods of increased temperature (i.e. heat waves) will occur more frequently and be more severe in the coming decades (Pachauri et al., 2014). Heat adversely affects plant growth and development and thus, in order to maintain food security, there is an urgent demand to adapt crop plants to the changing climate. While extremely high temperatures affect many basic physiological processes, reproduction-related processes, particularly pollen development, are vulnerable already to more mildly elevated temperature, especially if this is maintained for multiple days (Hedhly et al., 2009; Müller and Rieu, 2016). This also applies to tomato, an important vegetable crop and model plant for the large Solanaceae family (Peet et al., 1998; Pressman et al., 2002).

Pollen development occurs within the anther locule, which is surrounded by a layered wall consisting, from inside to outside, of tapetum, endothecium, middle layer and epidermis, respectively (McCormick, 2004). Under normal conditions, the generative pollen mother cell (PMC) undergoes meiosis to produce four haploid spores in the form of a tetrad, which subsequently separates to release free microspores. Microspores then undergo vacuolization, mitosis, and at the end give rise to mature pollen grains (McCormick, 2004). Heat-induced male sterility is described to have various causes, probably dependent on the used plant species and, especially, temperature regimes. In principle, it can be due to the susceptibility of the developing pollen cell itself, or to that of the surrounding sporophytic tissues. Temperatures higher than 35°C, for example, directly affected recombination and cytokinesis during meiosis of PMCs (De Storme and Geelen, 2014). Due to the dependence of developing pollen on the surrounding tissues, heat-induced alterations to the anther wall were also linked with pollen failure. The tapetum, as the innermost wall layer, nurtures the pollen cells during development by providing nutrients, enzymes and wall components (Pacini et al., 1985). At late microspore stage, the tapetum undergoes degeneration, a process that is tightly regulated and associated with successful pollen development (Wu and Cheung, 2000). Male sterility was found in numerous mutants with compromised tapetal function or degeneration (Parish et al., 2012). In several species, high temperature was suggested to impact pollen by compromising tapetal development or functioning (Abiko et al., 2005; Porch and Jahn, 2001; Saini et al., 1984; Suzuki et al., 2000). In addition to tapetum, stamium was also reported to be affected by heat: it failed to degenerate at anthesis stage, leading to reduced or impaired anther dehiscence and pollen shedding (Satake and Yoshida, 1978; Sato et al., 2002).

In tomato, the mechanism by which multiple days of MH lead to male sterility is still not well understood. Like with short heat shock, long-term mildly elevated temperatures around the meiosis to early microspore stage greatly reduced pollen number and viability.
(Iwahori and Takahashi, 1964; Rudich et al., 1977; Sato et al., 2002). It is not clear, however, what developmental process or tissue is primarily affected by the high temperature. Iwahori (1965) reported that after a short intense heat treatment (i.e. 2 days with 3 h at 40°C), tetrads appeared to be shrunken, and several days later developing microspores appeared to be dead. At the same time, tapetal cells showed developmental aberrations, growing larger during development instead of being degraded. Analysis of mature anthers that completely developed under long-term MH (i.e. 32°C/26°C, day/night) showed, in addition to dead pollen, reduced stomium cell degradation and structural abnormalities of the anther wall (Sato et al., 2002). In a similar setup, sugar metabolism was shown to be different at the late stages of pollen development (Firon et al., 2006; Pressman et al., 2002). Gene expression studies also revealed, in general, genes belonging to classical protective heat stress response (HSR), the processes of carbohydrate metabolism and oxidation-reduction regulation were transcriptionally up-regulated in anthers with meiosis to early and developing microspores under mild and severe heat stress (Bita et al., 2011; Frank et al., 2009; Li, 2015). However, all these studies analysed plant tissues in the first hours of heat stress and thereby were targeted primarily at the immediate heat response. In contrast, little is known about the long-term effects of damaging heat treatments on anther and pollen developmental pathways.

In the present work, we examined the effects of multiple days of MH on pollen and tapetum development in tomato. We defined a window of MH sensitivity and minimum effective treatment. Then, developmental abnormalities were identified through a detailed cytological study over time and transcriptome analysis on the day after the end of the minimal MH treatment.

**Materials and methods**

**Plant growth management and treatment**

*Solanum lycopersicum* cultivar Micro-Tom was obtained from the “National BioResource Project (NBRP)”, Japan (accession TOMJPF00001). Seeds were sown on normal commercial potting soil (Lentse Potgrond number 4, Horticoop B.V., Katwijk, The Netherlands), covered with a layer of vermiculite and kept in a cabinet under control condition. Two weeks later, seedlings were transferred into the same soil supplemented with 4 g L⁻¹ fertilizer (Osmocote exact standard 3-4 M, Evris International B.V., Geldermalsen, The Netherlands). One month after sowing, plants were subjected to either control or MH condition. All experiments were done with a photoperiod of 12h/12h (day/night), light intensity of 200 µmol s⁻¹ m⁻² (Green Power LED DR/B/FR 120 lamps, Philips, Eindhoven, The Netherlands) and 60% relative humidity. For the initial experiment to
Mild heat during early flower development results in pollen abortion at late stages.

determine the sensitive stage of tomato pollen development to MH with a heat treatment-release approach, control and MH were set at 22°C/22°C, 34°C/22°C (day/night), whereas all subsequent analysis were done at 25°C/19°C for control and 33°C/27°C for MH.

**Pollen viability assay**

The pollen viability was determined by an *in-vitro* germination assay. In brief, anthers from freshly opened flowers were cut into 4 slices and rehydrated in 1.5 mL Eppendorf tubes for 30 min. Afterwards, pollen were incubated for 1.5 h in 0.5 mL germination medium (25% (w/v) PEG 4000, 5% (w/v) sucrose, 1 mM KNO$_3$, 1 mM Ca(NO$_3$)$_2\cdot4$H$_2$O, 1.6 mM H$_3$BO$_3$, 0.8 mM MgSO$_4\cdot7$H$_2$O) under constant rotation. For every flower, 10 µL pollen suspension was loaded onto a haemocytometer for counting. Pollen with tubes longer than the diameter was considered as germinated.

**Sampling, embedding and microscopy for cytological analysis**

To look at the developmental changes caused by MH, anthers were collected from control and 4-day MH (from 13 to 10 DBA) treated plants, at 9, 8, 6, 5, 3, 1 and 0 DBA (see Figure 1 for experimental setup). Afterwards, anthers were fixed in 0.025 M phosphate buffer, pH 7.2, containing 2% glutaraldehyde and 4% paraformaldehyde, for 2 h at room temperature, followed by 2 h post fixation with 1% osmium tetroxide in water. The samples were then dehydrated in a graded ethanol series and embedded in Spurr’s resin. For light microscopy, sections of 1 µm in the median part of the anthers were cut and stained with 0.1% toluidine blue in 1% borax. Sections were viewed and photographed with a Leica DM2500 microscope (Leica Microsystems GmbH, Wetzlar, Germany), equipped with a Leica DFC 420C camera. For electron microscopy, 70 nm sections were cut and post-stained with uranyl acetate and lead citrate according to standard procedures and viewed with a JEOL JEM-1010 equipped with a Mega View III from Soft Imaging System.

In order to compare the developing pollen from control and MH, the number of cells per developmental stage (as described in supplementary table S1) was counted in each locule. Pollen was classified as with normal appearance, aberrant in shape and size, plasmolysed or dead (i.e., no cytoplasm or only remnants of degenerated cytoplasm was present). The tapetum stages were classified as described in supplementary table S2. Because tapetum cells of different stages were usually present in one locule, the proportions of the stages were scored and mean value was used. In total, we analysed 770 locules containing 47822 developing pollen from control condition and 758 locules containing 45016 developing pollen after heat treatment. For each time point, the anthers of 5-7 flowers were analysed.

For the comparison of pollen size between control and 4-day MH, fresh pollen was viewed and photographed with a Leica DM2500 microscope and Leica DFC 420C camera.
after staining with 0.1% toluidine blue in 0.1% borax. In total, 2260 (11 flowers) from control and 1874 (9 flowers) from 4-day MH were analysed by using the image analysis software FIJI (https://imagej.nih.gov/ij/). Area of pollen with a round shape was determined.

**Figure 1. Experimental setup for cytology and microarray analysis.**

A) Coordination between pollen developmental stages and DBA by referring the cytological study with control samples. B) 1- and 4-day MH were imposed until the end of 10 DBA. Ten flower buds in early tetrads stage were tagged from both, control (3.1 mm) and 4-day MH (3.0 mm), and these reference buds were used to determine the size of flower buds sampled for cytology on subsequent days. No clear differences were observed between control and 4-day MH. C) For microarray analysis, flower buds were only collected on 9 DBA, while samples at 7 time points were used for cytology.

**Sampling, RNA extraction and microarray analysis**

Samples for microarray analysis were taken from plants recovered at 25°C for 3 h after the last night of MH treatment. Anther tissue of 3.6-3.7 mm flower buds (9 DBA) were collected for total RNA isolation with Trizol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA), followed by DNA digestion and purification with an RNeasy kit (Qiagen, Venlo, The Netherlands). RNA concentration and quality were checked by Nanodrop 1000 (ThermoScientific, Thermo Fisher Scientific) and 1.5% agarose electrophoresis. The microarray analysis (i.e. RNA quality check, cDNA synthesis, array hybridization, image analysis, data processing and Robust Multi-array Average normalisation) were conducted by the use of Affymetrix tomato EUTOM3 arrays at the Wageningen UR genomics facilities, in collaboration with Dr A. G. Bovy. Original expression data were filtered for absolute
Mild heat during early flower development results in pollen abortion at late stages

maximum expression value (≥20) before gene set enrichment analysis (GSEA, version 2.2.2, Broad Institute, MIT) with pre-defined MapMan bins (http://www.gomapman.org; Ramsak et al., 2014) and manually-generated gene sets. The latter consisted of heat stress transcription factors (HSFs), heat shock proteins (HSPs), HSR genes (Fragkostefanakis et al., 2015), sugar metabolism, pollen and tapetum development, unfolded protein response (UPR) induced genes (Fragkostefanakis et al., 2016), up- or down-regulated genes in immature tomato anther upon heat shock (Li., 2015), and genes for reactive oxygen species (ROS) scavenging (http://biol.unt.edu/~rmittler/genelist.htm). The significantly enriched MapMan bins were determined by false discovery rate (FDR) with cut-off value of 0.1 and visualized as a heatmap. A pathway regarding sucrose and starch metabolism was adapted from two studies (Ferreira and Sonnewald, 2012; Mangelsen et al., 2011) and genes responsible for different reactions (http://solcyc.solgenomics.net/) were mapped in the pathway.

Furthermore, the expression data filtered by maximum expression value were filtered again by the median of inter quantile range (based on log_2 value) to identify differentially expressed genes (DEGs) via a one-way ANOVA followed by Tukey’s Post Hoc test, as implemented with in-house R scripts. Two comparisons, 4-day MH versus control and 1-day MH versus control were made to investigate the effects of MH on transcriptional response. Moreover, 4-day MH was compared with 1-day MH and control to identify pollen phenotype associated genes. The FDR method was used to control false positives due to multiple testing (Benjamini and Hochberg, 1995) and q value of 0.1 was set as cut-off for ANOVA. Statistical over-representation test from PANTHER (http://pantherdb.org; Mi et al., 2013) was employed to identify enriched gene ontology (GO) terms in the sets of up- or down-regulated DEGs from all comparisons. A Bonferroni-adjusted P-value of 0.05 was set as cut-off for significant enrichment.

Statistical analysis

The pollen viability data were analysed by one-way ANOVA and Tukey’s Post Hoc test which are implemented with SPSS (v20, IBM, NY, USA). For the experiment to determine the sensitive window to MH treatment (Figure 2A), means were labelled with letters to show significant differences. In the experiment for MH durations’ effects (Figure 2C) and experimental setup for cytology and microarray (Figure 2D), values from MH treatments were compared with that from corresponding control samples.
Results

Characterization of tomato pollen viability under MH

To determine the sensitive stages of tomato pollen to MH, plants were subjected to a 2-week period of MH, followed by a 2-week release at control temperature. Pollen viability was determined by *in-vitro* pollen germination assay with flowers at anthesis-stage collected daily. Under MH, a sharp decrease of pollen viability was seen if the flowers were treated at least from 9 DBA. In the subsequent release period, the pollen viability suddenly increased from 20 to 50% if flowers were developed under control temperature for at least 11 days (Figure 2A). This indicates that the period of 9-11 DBA is highly sensitive to MH.

To determine the minimal treatment duration necessary to see clear effects on pollen viability, periods of MH with different lengths (2, 3 and 4 days) were applied (see experimental setup in Figure 2B). In comparison with control, substantial reductions (from about 50% to 20%) were observed from plants which were stressed for 3 and 4 days MH until ~11-7 DBA. By contrast, 2 days MH only caused about 10% decrease in viability (Figure 2C). To confirm the contrasting phenotypic responses to short (≤ 2 days) and long (≥ 3 days) MH periods, 1- and 4-day MH treatments were tested again, pollen viability declined sharply during the sensitive stages after 4-day MH, whereas 1-day MH did not impose significant effects on tomato pollen (Figure 2D).

Consequences of 4-day MH on pollen development

As 4-day MH ending at 11-6 DBA was sufficient to substantially reduce viability of mature pollen (Figure 2D), cytological comparisons were made between anthers treated with MH for 4 days, from 13 to 10 DBA, and anthers kept at control temperature. Very little differences were found between the two treatments in the first five days after the end of the treatment. One day after the treatment, namely at 9 DBA, the MH exposure led to slightly higher variation in terms of pollen developmental stages, as cells in meiotic and later microspore stages were not observed in flowers from control condition (Figure 3). Also, in comparison to control, slightly higher proportions of dead pollen (in the late microspore stage) were found in 4-day MH treated anthers at 6 and 5 DBA (Figure 3). Independent of the treatment, between 6 and 5 DBA mitosis occurs, meaning most microspores developed into early binucleate pollen. At 3 DBA, some differences became apparent. While in both treatments, pollen has proceeded to the late binucleate stage, a larger proportion had already entered the mature stage in control than in the 4-day MH flowers. This trend was still seen at 1 DBA, where ~20% of the living pollen cells from the 4-day MH treatment were still at the binucleate stage, compared to only 1% of control pollen. Much more striking, however, was that some binucleate pollen from 4-day MH (6%) became aberrant (i.e. oval shape or less dense cytoplasm) at 3 DBA and 50% of pollen appeared to be died at the binucleate stage.
Figure 2. The effects of MH timing and duration on tomato pollen viability.
A) 2-week MH and 2-week release experiment to identify the sensitive stage of pollen development to MH. Temperature was set at 34°C/22°C (day/night). Samples labelled with different letters differ significantly from each other, P<0.05. B) MH durations and sampling schedule for pollen analysis. Samples were coded according to the last day of MH treatment, i.e. “1 DBA” means that MH ended at 1 day before anthesis. C) Pollen viability from MH with different durations. D) Confirmation of phenotypic response under 1- and 4-day MH. This setup was used for cytological and transcriptomic analysis. For C, D: *, significantly different from control treatment, P≤0.05; **, P≤0.01; ***, P≤0.001. Values were the mean ± SE.
In contrast to the strong effect of 4-day MH on pollen development, no clear influence on the tapetum was observed. Although at one day after the treatment, the mean tapetum stage in control conditions was 5.3 compared to 6.2 in heat conditions, there was no suggestion of faster developmental progression in the following days (Figure S1), indicating that no major developmental deviations took place in the tapetum.

**Figure 3. Comparison of developing pollen from control and 4-day MH treatment.**

The MH was applied for 4 days starting at 13 DBA. Afterwards, samples were taken at 9, 8, 6, 5, 3, 1 DBA for cytological sections. Observations were made using light microscopy and developing pollen grains were assigned to 4 categories, namely dead, plasmolysed, aberrant shape and normal.

**Cytological consequences of 4-day MH on maturing pollen**

At 3 DBA, the binucleate stage started with the accumulation of starch. This process occurred in both, control and 4-day MH conditions, but was more variable in MH treated pollen, which often had lower numbers of starch granules (Figure 4A, D, 5A, B). Also, in MH pollen, starch granules tended to be smaller and form clusters (Figure 5B).
Mild heat during early flower development results in pollen abortion at late stages. The process was followed by the second vacuolisation, in which a large vacuole was formed, the starch disappeared and the size of the pollen gradually increased (Figure 4A, D, 5C). With the subsequent increase in cytoplasm and loss of the large vacuole, numerous new starch granules were formed towards the mature pollen stage. After the 4-day MH treatment, pollen development was less synchronised and again a proportion of pollen produced fewer and smaller starch grains (Figure 4B, E, 5D, E). At anthesis stage, starch was not present anymore in both control and MH stressed pollen (Figure 4C, F, 5F, G). No or only small vacuoles were left in control pollen, but in MH the size and number of vacuoles were more variable, often many larger vacuoles were observed and also the size of pollen seemed smaller than those from control temperature (Figure 4C, F, 5F, G). Analysis of the pollen area at this stage from fresh pollen samples confirmed this. Size of 4-day MH treated pollen area peaked around 500-600 µm² and only 10% of pollen had an area of 600-800 µm², whereas under control temperature, 33% of pollen had an area of 600-800 µm² (Figure 4G).

Figure 4. Light microscopy analysis of maturating pollen. A, D) Early binucleate pollen from control (A) and 4-day MH treatment (D). B, E) Mature pollen from control (B) and 4- day MH (E). C, F) Anthesis stage pollen from control (C) and 4-day MH (F). Bar=20 µm. G) Size of pollen at anthesis stage, measured as area in µm² from images of fresh pollen samples.
Transcriptional responses of young anthers to 4- and 1-day MH

To understand the molecular physiological basis of MH-induced pollen failure, we determined transcriptomic alterations caused by the 4-day MH treatment, which significantly reduces pollen viability, as well as by 1-day MH treatment, which did not significantly affect pollen viability. Developing anthers from 3.6-3.7 mm flower buds (Figure 1) were collected for microarray analysis 3 h after the end of the night still under the 1- or 4-day MH treatment. To study the behaviour of gene sets in whole gene expression dataset, GSEA was applied by the use of 386 gene sets that represent pre-defined MapMan bins. Thirty-six of them were significantly enriched in at least one of the three comparisons, namely 1- and 4-day MH versus control, 4-day MH versus 1-day MH. MapMan bins related with protein synthesis (29.2.1), protein folding (29.6) and salicylic acid (17.8) metabolism were negatively modulated by MH and more so with 4-day MH, whereas the negative effects of MH on cell cyle (31.3), DNA synthesis and metabolism (28.1.3) became less when MH period was increased (Figure 6A). By contrast, gene sets related to protein degradation (29.5.2, 29.5.3), cell wall modification (26.3.4, 10.6.1), secondary metabolism (16.1.4, 16.2.1, 16.1.5, 16.2.1.3) and lipid degradation (11.9.2.1, 11.9.2), as well as transcription factor genes (27.3.7, 27.3.8, 27.3.32) were found to be up-regulated (Figure 6A). No MapMan gene sets were significantly enriched in either direction in the comparison 4-day versus 1-day MH (Figure 6A).

GSEA was also conducted with several selected gene sets (Figure 6B). Overall, the
Mild heat during early flower development results in pollen abortion at late stages.

Figure 6. GSEA results of three comparisons among control, 1- and 4-day MH.
A. MapMan bins that were significantly affected in at least one of the three comparisons were visualized. The colour key: red means bins are up-regulated bins, blue means for down-regulation. CT: control, 1d: 1-day MH, 4d: 4-day MH. * (marginally) significant difference between the two treatments, P≤0.1; *, P≤0.05; **, P≤0.01; ***, P≤0.001.
HSR, UPR and pollen and tapetum development related gene sets were expressed at lower levels after MH treatment. In case of “heat shock induced genes in tomato anther” and “unfolded protein induced genes”, this down-regulation was significantly stronger after 4 days than after 1 day of MH. Sucrose cleavage and ROS scavenger were up-regulated by the 1-day MH treatments (Figure 6B), but in contrast to the latter, the sucrose cleavage was not induced by the 4-day MH treatment.

To look specifically at genes with significantly modified expression levels, one-way ANOVA followed by multiple testing correction identified 154 DEGs (FDR q value≤0.1) from the expression data set. All these genes where either significantly up- (69) or down-regulated (85) by 1- or 4-day MH compared to control, with most of them commonly modulated by both treatments (Figure 7A, B). Three high-level GO (GO-slim) of the biological process (BP) category were overrepresented among significantly down-regulated genes (i.e. protein folding, DNA replication, response to stress), indicating a coordinated transcriptional response to the treatment (Figure 7C).

Direct comparison of 4-day versus 1-day MH identified 15 significantly higher and 32 lower expressed genes (Figure 7D, E). These small groups were not enriched for any GO-slim BP term. To further discern changes in gene activity related to pollen phenotype, we filtered these genes to also be expressed significantly higher or lower after 4-day MH compared to control treatment. In total, the pollen failure phenotype was associated with up-regulation of 7 genes and down-regulation of 21 genes (Table 1). Notably, 9 of the later group were associated to the UPR (Table 1).

Figure 7. Venn diagrams of DEGs and over-representation test for gene ontology (GO) terms.
A, B) up- and down-regulated genes from two comparisons, 1-day MH versus control and 4-day MH versus control. C) significantly enriched GO terms from over-representation test. D, E) Another two comparisons, 4-day MH versus both control and 1-day MH to identify pollen phenotype associated genes. CT: control, 1d: 1-day MH, 4d: 4-day MH.
Mild heat during early flower development results in pollen abortion at late stages.

Table 1. Functional characterization of commonly regulated genes from 4-day MH versus both control and 1-day MH.

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Table 1. Functional characterization of commonly regulated genes from 4-day MH versus both control and 1-day MH. (continued)

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Commonly down-regulated genes from 4d VS CT and 4d VS 1d 1

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| Solyc01g104760      | Homeodomain-like                                | AT1G76870 |           |                 |
|                     |                                                | AT1G21200 |           |                 |
| Solyc03g120390      | Auxin responsive protein IAA15                 | AT1G80390 | IAA15     |                 |
| Solyc08g078960      | Oxysterol-binding family protein                | AT4G22540 | ORP2A     |                 |
|                     |                                                | AT4G12460 | ORP2B     |                 |
| Solyc12g056560      | Alpha-crystallin domain of heat shock protein-containing protein | AT1G76780 |           |                 |
|                     |                                                | AT1G76770 |           |                 |
| Solyc07g052490      | Myb family transcription factor (unknown)      |           |           |                 |
| Solyc02g091750      | Starch synthase V (unknown)                     |           |           |                 |

1CT: control, 1d: 1-day MH, 4d: 4-day MH.
2Arabidopsis orthologs corresponding to tomato genes.
3The Arabidopsis orthologs were labeled with “Y” if they are induced by UPR.

Since the cytological study revealed morphological alterations of starch granules, we looked in more detail at genes related to sucrose and starch metabolism. Nearly all enzymes in the pathway displayed a similar expression pattern after 1- and 4-day MH treatment, except for those involved in sucrose cleavage (cell wall invertase, vacuolar invertase, sucrose synthase, figure 8), which were up-regulated by 1-day MH, but less induced or even reduced after the 4-day MH treatment, and those involved in the last two steps of starch synthesis (ADP-glucose pyrophosphorylase, starch synthase) showed a mixture of up- and down-regulation by 1- and 4-day MH (Figure 8).
Figure 8. Transcriptional overview of genes involved in sucrose and starch metabolism. The pathway was adapted from Ferreira and Sonnewald (2012) and Mangelsen et al. (2011). Genes responsible for each enzyme were retrieved from (http://solcyc.solgenomics.net/); only expressed genes were shown. AGPase: ADP-glucose pyrophosphorylase, CIN: cytosolic invertase, CWIN: cell wall invertase, FK: fructose kinase, HK: hexose kinase, PGI: glucose-6-phosphate isomerase, PGM: phosphoglucomutase, SBE: starch branching enzyme, SS: starch synthase, SuSy: sucrose synthase, SUT: sucrose transporter, UGPase: UDP-glucose pyrophosphorylase, VIN: vacuolar invertase. Colour key represents for Log(fold change) of the indicated comparisons (CT: control, 1d: 1-day MH, 4d: 4-day MH); red means for up-regulation, blue for down-regulation. #, (marginally) significantly different between the two treatments, P≤0.1; *, P≤0.05; **, P≤0.01; ***, P≤0.001.

Discussion

A window of sensitivity to MH during tomato pollen development

In the present work, the MH, as a proxy for heat wave conditions, was shown to negatively affect tomato pollen viability, in line with several other reports (Peet et al., 1998; Pressman, 2002). Pollen development is a complex sequence of processes, starting from the pollen mother cells, which, after meiosis and mitosis, give rise to binucleate mature pollen (McCormick, 2004). In a previous study, the application of MH defined the sensitivity window of developing flowers as 15-7 DBA (Sato et al., 2002), which encompasses the...
shorter period of highest susceptibility identified here, i.e. from 11-9 DBA. This latter window corresponds to meiosis to microspore transition phase during pollen development. High sensitivity around meiosis was also found for other crops and other abiotic stresses (De Storme and Geelen, 2014). Interestingly, sensitivity to a heat shock of 3 h at 40°C on two subsequent days and to a “one hot day” treatment of 10 h at 38°C were also reported to peak at the meiosis to microspore transition (Iwahori, 1965; Müller and Rieu, personal communication), suggesting that severe heat and MH, and possibly other stress factors, affect developing pollen via the same mechanism. As MH requires exposure for multiple days to have a clear phenotypic effect, pollen development may respond to the heat sum (temperature x time, also known as thermal time).

Cytological effects of early MH stress become apparent in maturing pollen
Pollen development takes place inside the locule, surrounded by several layers of sporophytic tissues, namely tapetum, middle layer, endothecium and epidermis (McCormick, 2004). While anthers exposed to heat during their development are characterised by production of pollen with reduced viability, at earlier stages, heat can also induce alterations in surrounding cell layers, particularly the tapetum. Under normal conditions, the tapetum undergoes degradation through programmed cell death at late stage of unicellular microspores (Wu and Cheung, 2000). Premature degeneration of tapetum, however, was observed upon heat stress in several plant species (Abiko et al., 2005; Harsant et al., 2013; Saini et al., 1984; Suzuki et al., 2000) and suggested to be causally connected to pollen failure (De Storme and Geelen, 2014; Parish et al., 2012). In the present work, 4 days of MH treatment (from 13 to 10 DBA) resulted in a ~30% pollen viability, which is about half of that found under control conditions, but strikingly, no clear cytological changes in the developing pollen and tapetum were observed for the first week after the end of the treatment (i.e. until 3 DBA). Only after pollen mitosis, between 3 and 1 DBA, suddenly a large proportion of the binucleate pollen died. Thus, the binucleate pollen stage may has a specific requirement, which is not fulfilled if developing anthers are exposed to MH earlier on.

Protein folding after MH exposure
One of the hall-mark effects of high temperature on living organisms is incorrect folding of proteins. Correspondingly, a highly conserved HSR exist that includes activation of chaperones, which prevent misfolding and promote refolding of proteins in cytoplasm, plastids and mitochondria (Al-Whaibi, 2011; Wang et al., 2004). A second set of genes, also including those encoding chaperones, is induced to protect against toxic levels of unfolded proteins in the endoplasmic reticulum (ER), in the so-called UPR (Fragkostefanakis et al., 2016; Howell, 2013; Iwata and Koizumi, 2012). During or shortly after a heat shock or MH, the HSR and UPR were up-regulated, also in tomato reproductive tissues (Bita et al., 2011;
Mild heat during early flower development results in pollen abortion at late stages

Frank et al., 2009; Chaturvedi et al., 2015; Fragkostefanakis et al., 2016; Li, 2015). By contrast, in our samples taken after 1-day MH, i.e. 3 hours after the 25°C night, but already 15 hours after the higher 33°C daytime period, many of the HSR and UPR genes showed decreased expression and, especially UPR-related genes, and an even lower expression after 4-day MH. The ER of the tapetum, which is essential for pollen development-related processes, is thought to be highly sensitive to environmental stress, including high temperature. Because UPR gene expression responds to a signalling pathway starting in the ER itself, its reduction may be the result of heat induced dysfunction of tapetal ER (De Storme and Geelen, 2014; Suzuki et al., 2000). Interestingly, recent studies indicated an intact UPR to be essential for pollen development under very mildly elevated or even normal temperatures (Deng et al., 2016; Fragkostefanakis et al., 2016; Reyes et al., 2010; Yang et al., 2009). However, the strong down-regulation of HSR and UPR genes in early microspore stage anthers after MH may also reflect negative feedback, where a surplus of protective proteins in the absence of heat results in below-normal levels of unfolded-protein stress and down-regulation of the response pathway (Hahn et al., 2011; Kim and Schöffl, 2002; Morimoto, 1998). The same phenomenon was recently observed in grape leaves (Liu et al., 2012). As yet it is not clear if the basal level of HSR and UPR gene expression in anthers is stress-dependent and thus subject to feedback, or developmentally determined as has also been suggested by Chaturvedi et al. (2016) and Fragkostefanakis et al. (2016).

A potential role for sugar metabolism in MH-induced pollen defects

Starch and soluble sugar levels are finely regulated during pollen development; under normal conditions, starch accumulates and suddenly reaches a peak after pollen mitosis I, followed by gradual break-down into soluble sugars at anthesis stage (De Storme and Geelen, 2014). These simple sugars then serve as the energy source for imminent pollen germination. Reduced starch accumulation has been associated with male sterility upon various abiotic stresses (Dorion et al., 1996; Oliver et al., 2005; Sheoran and Saini, 1996). In tomato, treatment with MH during the complete period of flower development significantly decreased the starch concentration in binucleate pollen and, consequently, lowered the level of soluble sugar content at anthesis stage (Firon et al., 2006; Pressman et al., 2002; Sato et al., 2006). Our results confirm and extend these findings by showing that a 4-day MH around meiosis to early microspore stage was sufficient to negatively affect starch deposition in binucleate pollen. Notably, we observed two distinct instances of starch accumulation in binucleate pollen, interrupted at the so-called second vacuolisation phase; in both instances, 4-day MH resulted in fewer and smaller starch granules. As carbohydrates are major osmolytes in pollen, reduced increase in pollen volume during the final developmental stages may also be the result of reduced soluble sugar content.
(Firon et al., 2012). The relation between carbohydrate content and pollen viability was corroborated by the finding that more tolerant genotypes were able to maintain starch and sugar levels better than sensitive genotypes (Firon et al., 2006). However, these results have not clarified whether low carbohydrate levels are part of the cause of MH-induced pollen abortion or rather are a symptomatic consequence of reduced pollen functioning. We found that a number of genes responsible for sucrose and starch metabolism responded to the MH treatments in early microspore stage anthers, several days before the first negative effects of 4-day MH on pollen morphology and the carbohydrate profile become evident. A sucrose transporter gene was highly upregulated by both treatments. More interestingly, several genes encoding sucrose cleavage enzymes, i.e. cell wall invertase (CWIN), vacuolar invertase (VIN) and sucrose synthase (SuSy), responded differently to the two MH treatments: they were induced by the non-damaging 1-day MH, but not by the damaging 4-day MH treatment. Of these, the invertases seem to be the most important in anthers, and especially CWIN was known to play a crucial role in sucrose unloading into the symplastically isolated microspores (Goetz et al., 2001; Castro and Clement, 2007; De Storme and Geelen, 2014). Indeed, silencing of a tomato CWIN was found to significantly reduce pollen viability (Zanor et al., 2009). Since usually there is a high cellular energy demand to recover from exposure to abiotic stressors (Avin-Wittenberg et al., 2012; Ghosh and Xu, 2014; Jacoby et al., 2011), the observed higher level of CWIN after 1-day MH might act to supply the necessary energy to reestablish cellular homeostasis. By contrast, the non-induced level after 4-day MH, might not be sufficient to drive recovery. Pressman et al. (2006) even reported down-regulation of CWIN gene and enzyme activity in late microspore-stage anthers of tomato under long-term MH. The expression pattern of VIN was similar to that of CWIN and also this gene was reported to be down-regulated by continuous MH in meiotic and microspore stage anthers (Sato et al., 2006). Recently, it was shown that silencing of VIN in reproductive organs could also lead to reduced pollen viability (Wang and Ruan, 2016). In addition, we found several starch synthase genes to be differentially affected by the two MH treatments, but no clear pattern could be discerned among them. Carbohydrate partitioning between the different cell types in the anther depends on a dynamic metabolic network (De Storme and Geelen, 2014), so it will be important to localise the expression of the genes found to be affected by MH. CWIN genes, for example, are thought to function either in the developing pollen, or tapetum, or both (De Storme and Geelen, 2014). While tapetum development upon 4-day MH did not deviate from control regarding gross morphology, its physiology and gene expression activity may still be affected. Endo et al. (2009) described a similar situation in rice under high temperature, with clear gene expression changes in the tapetum, despite a normal developmental progression.

In conclusion, we have shown that MH damages early pollen development and that this
Mild heat during early flower development results in pollen abortion at late stages first becomes apparent several days later, after pollen mitosis. Notably, we did not detect differential expression of known transcriptional regulators of pollen or tapetum development after MH treatments. Reduced UPR protein levels or reduced sugar availability might play a role in MH-induced pollen failure, which should be tested by modifying these systems in vivo.

Acknowledgement

The authors would like to thank Dr Liesbeth Pierson for help with image analysis to determine pollen size and Nicky Driedonks for harvesting samples used for microarray experiment.
Chapter 2

References


Mild heat during early flower development results in pollen abortion at late stages


48. Reyes, F., León, G., Donoso, M., Brandizzi, F., Weber, A.P.M., Orellana, A., 2010. The nucleotide sugar transporters AtUTr1 and AtUTr3 are required for the incorporation of UDP-glucose into the endoplasmic reticulum, are essential for pollen development and are needed for embryo sac progress in Arabidopsis thaliana. Plant J. 61, 423–435. doi:10.1111/j.1365-313X.2009.04066.x
Mild heat during early flower development results in pollen abortion at late stages

## Supplementary tables and figure

Table S1. Pollen developmental stages and corresponding description.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-meiotic</td>
<td>Differentiation of Pollen Mother Cell (PMC) and tapetum which is in angular shape. Nucleus with nucleolus and small vacuoles are visible.</td>
</tr>
<tr>
<td>Meiosis</td>
<td>Meiotic division until Anaphase II. PMCs become less angular and tend to be round. Callose deposition around PMCs. Separation of tapetum starts.</td>
</tr>
<tr>
<td>Tetrads</td>
<td>From the end of meiotic division, telophase II and cytokinesis until the start of callose dissolution. Callose deposition between microspores and formation of tetrads.</td>
</tr>
<tr>
<td>Early Microspore</td>
<td>Callose degradation leads to release of free microspores in which apertures are visible. Microspore cell wall is thickening. Irregular shaped microspores slowly turn spherical.</td>
</tr>
<tr>
<td>Late microspore</td>
<td>Microspores are spherical and larger. Pores become clearly visible. Microspores undergo vacuolisation, forming numerous (&gt;5) vacuoles that will merge, forming one large vacuole.</td>
</tr>
<tr>
<td>Binucleate</td>
<td>Mitotic division give rise to two cells. At early binucleate, generative cell is appressed to pollen wall and smaller than vegetative nucleus. Inside developing pollen, smaller and irregular vacuoles are visible. During second vacuolisation, Large vacuoles will form and fuse into one large vacuole. Afterwards, accumulation of starch, intine thickening at apertures. Generative cell is free in cytoplasm.</td>
</tr>
<tr>
<td>Mature</td>
<td>Mature pollen as large spherical grains. Accumulation of starch around nucleus, numerous lipid droplets and small vacuoles can be present.</td>
</tr>
<tr>
<td>Anthesis</td>
<td>Spherical pollen, starch has disappeared. Black inclusions in cytoplasm, lipid droplets are prominent. Faint nucleolus.</td>
</tr>
</tbody>
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Supplementary tables and figure

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<td>Differentiation Microspore Mother Cell and tapetum. Small angular cells with large nucleus, as well as small and large vacuoles.</td>
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<td>Binucleate tapetum cells. Cells become elongated and contain more small vacuoles</td>
</tr>
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<td>3</td>
<td>The tapetum separates from pollen mother cells. Cell wall (locule side) of tapetal cells is dense stained.</td>
</tr>
<tr>
<td>4</td>
<td>Larger vacuoles with dense stained deposits. Outer walls at the locule side appear to be thicker and more densely stained.</td>
</tr>
<tr>
<td>5</td>
<td>Tapetum cells become irregular shaped and cell walls are weakly stained. Sometimes, bluish line at the locule side visible. Few small round orbicules deposited on the walls at the locular side and in between the tapetum cells. Some small grey droplets in the cytoplasm are visible, but hardly to see the nucleoli.</td>
</tr>
<tr>
<td>6</td>
<td>Large amounts of round orbicules deposited on the walls at the side of the locule and in between tapetum cells. In vacuoles and cytoplasm, larger grey droplets are observed, but hardly to find nucleoli.</td>
</tr>
<tr>
<td>7</td>
<td>Start of degeneration. Signs of degeneration: solid dense stained cytoplasm, nucleus dissolving, vacuoles aggregate and become larger. Cells show strong plasmolysis.</td>
</tr>
<tr>
<td>8</td>
<td>The tapetum cells are degrading and cytoplasm becomes less dense stained. Large grey-brown spots are clearly visible. Small amount of remnants of dense stained cytoplasm are still present.</td>
</tr>
<tr>
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<td>All tapetum cells show remnants of degraded cytoplasm. Nuclei are disintegrated and Large grey-brown spots are visible.</td>
</tr>
<tr>
<td>10</td>
<td>Only packed cell walls left with remnants of large grey-brown spots and dense deposits, no remnants of cytoplasm.</td>
</tr>
<tr>
<td>11</td>
<td>Packed cell walls with hardly any remnants of grey-brown spots and dense deposits.</td>
</tr>
</tbody>
</table>

Table S2. Tapetum developmental stages and corresponding description.

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Figure S1. Comparison of tapetum development from control and 4-day MH.
After the 4-day MH treatment, samples were taken on 9, 8, 6, 5, 3, 1 DBA. Since the tapetum development is correlated with pollen stages, the mean value of tapetum developmental stage (developmental stages are variable at the same pollen stage) was compared between control (open bars) and 4-day MH (black bars), X axis label indicates the pollen stage in anther locules. MMC: microspore mother cell.
The involvement of reactive oxygen species in tomato pollen heat tolerance

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Abstract

The damage of heat on plants is ascribed in part to accumulation of reactive oxygen species (ROS). While this has been established for vegetative tissues, much less is known about the role of ROS in heat damage of reproductive tissues. Pollen development is particularly prone to heat, and even a few days of mildly elevated temperatures can drastically reduce viability of produced pollen. We used several approaches to test the role of ROS in mediating the heat effect on pollen development. Expression and enzyme activity of several ROS scavengers was enhanced during and after a mild heat (MH) treatment for 4 days, suggesting a higher demand for antioxidant capacity due to elevated ROS levels. However, we were unable to measure ROS levels, and found no changes in membrane lipid peroxidation levels of the anther as a whole. Still, increasing antioxidant level through melatonin application or lowering protein oxidation damage by ectopic expression of Arabidopsis glutaredoxin (AtGRXs17) in tomato improved pollen thermotolerance. Thus, there are indications for a role of ROS in pollen heat sterility, but more evidence is needed to draw strong conclusions.
Introduction

Plant have an optimum ambient temperature range, and higher than optimal temperatures impose negative effects manifested by reduced cellular functioning, impaired growth and development, and, in more extreme cases, cellular and organismal death (Bokszczanin et al., 2013; Wahid et al., 2007). The damage of heat is ascribed in part to accumulation of reactive oxygen species (ROS) which are inevitably produced as by-products during aerobic metabolism, deriving from different cellular compartments, including mitochondria, chloroplasts, peroxisomes and the apoplast (Apel and Hirt, 2004; Asada, 2006; Rhoads et al., 2006; Sagi and Fluhr, 2006). By contrast, ROS also act as important signal molecules mediating stress tolerance (Baxter et al., 2014; Suzuki et al., 2013a). Cells possess an extensive ROS scavenging machinery, which consists of enzymes such as catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and so on (Mittler, 2002; Mittler et al., 2004). Many of the ROS scavenging-related genes are responsive to ROS levels, which results in a finely regulated balance between production and removal under steady state conditions. Exposure to heat, however, can disturb this balance. A number of studies in vegetative tissues and cell types showed that heat rapidly led to the accumulation of ROS, resulting in a secondary, oxidative stress (Dat et al., 1998; Vacca et al., 2004; Volkov et al., 2006). Indeed, to cope with the excessive amount of ROS upon heat, the expression of ROS scavengers is rapidly up-regulated, too. For example, 1 h at 38°C induced the expression of \textit{APX1} and \textit{APX2} in \textit{Arabidopsis} (Suzuki et al., 2013b). Higher enzymatic activity of APX and CAT were also observed after relatively short periods of heat treatment in Kentucky bluegrass (He and Huang, 2010) and tobacco BY-2 cells (Sgobba et al., 2015).

In line with this, vegetative heat tolerance, measured as the ability to maintain chlorophyll content, was correlated positively with antioxidant enzyme activity in different wheat and bluegrass genotypes (Almeselmani et al., 2006; Du et al., 2013).

ROS have an important role in the formation of male gametophyte, the pollen. Developing microspores are symplastically isolated from the sporophyte and depend on the surrounding cell layer, the tapetum, for supply of carbohydrates, compounds and enzymes (Pacini et al., 1985; Wu and Cheung, 2000). During microspore development, the tapetum cells undergo programmed cell death, which involves ROS action. Arabidopsis and rice mutants with reduced amounts of ROS could not timely activate the programmed cell death of tapetum, leading to pollen failure (Xie et al., 2014; Yi et al., 2016). On the other hand, excessive ROS at the late microspore stage in rice \textit{mads3} mutants caused pollen sterility (Hu et al., 2011). Thus, tight regulation of ROS content is essential for production of viable pollen and reproductive success.

Pollen development is strongly affected by episodes of MH (Chapter 2; Ahmed et
al., 1992; Saini and Aspinall, 1982; Sato et al., 2002), but the physiological mechanism underlying heat-induced pollen failure is not clear. In rice, the expression of several ROS-related genes was highly induced in heat stressed florets (Zhang et al., 2012) and in the developing pollen of tomato, APX3 was highly expressed following 45°C heat shock (Frank et al., 2009), which may indicate the occurrence of ROS accumulation. Based on these observations and the fact that vegetative heat tolerance is intimately related to ROS homeostasis, we hypothesize that ROS play a role in heat-induced pollen defects. In this work, we assessed ROS-related gene expression and enzyme activity, as well as the damage of ROS on lipids after a 4-day MH treatment applied during the sensitive meiosis to early microspore stages of pollen development. In addition, we tested the effect of chemical and transgenic modification of the ROS system on pollen thermotolerance.

Materials and methods

Plant cultivation and mild heat treatment

*Solanum lycopersicum* cultivar Micro-Tom was obtained from the “National BioResource Project (NBRP)”, Japan (accession TOMJPF00001); the tomato lines ectopically expressing *AtGRXs17* and the corresponding wild type, cultivar Rubicon, were obtained for Dr Sunghun Park (Kansas State University, Manhattan, KS, USA, Wu et al., 2012). Seeds were sown on commercial soil (Lentse Potgrond number 4, Horticoop B.V., Katwijk, The Netherlands) and covered a thin layer of vermiculite. Two weeks after sowing, seedlings were transplanted into separate pots filled with the same soil (supplemented with 4 g L\(^{-1}\) Osmocote exact standard 3-4 M, Everris International B.V., Geldermalsen, The Netherlands). In experiments with Micro-Tom, during germination and seedling growth period, plants were always kept in cabinets under control condition (temperature: 25°C/19°C, day/night; light period: 12h/12h, day/night, 200 µmol s\(^{-1}\) m\(^{-2}\) provided by Philips Green Power LED DR/B/FR 120 lamps; 60% relative humidity). One month after sowing, plants were divided into 2 groups, with one maintained at control temperature and the other one subjected to the 4-day MH (33°C/27°C, day/night). Flower buds that received the MH at 13 to 10 days before anthesis (DBA) were used to collect anther samples at 9 time points (Figure 1, also see Figure 1 from chapter 2 for the length of flower buds). Per plant, anthers of multiple flowers were pooled. Three replicates (i.e. plants) were used per treatment per time point for enzyme activity assay, and four replicates for lipid peroxidation measurement and gene expression analysis.

In order to test the effects of exogenously applied melatonin (M5250; Sigma-Aldrich, St Louis, MO, USA) on tomato pollen thermotolerance, Micro-Tom plants were sprayed with melatonin 1 h or 24 h before the 4-day MH treatment. Freshly opened flowers were collected at day 7 and 8 after the heat treatment for pollen viability determination. To
understand the molecular response to melatonin, anther samples were collected from 1 h MH treated plants which were also sprayed with melatonin 1 h before start of MH.

For experiments with the AtGRXs17 lines, plants were grown under standard greenhouse conditions with 16-hour light period (supplemented with artificial light from 600W sodium lamps if natural light intensity fell below 250 µmol m$^{-2}$ s$^{-1}$) and temperature of about 25°C in the day (minimum set to 20°C) and 19°C in the night (minimum set to 17°C). When the first inflorescences were detectable by eye, all inflorescences were removed and plants were transferred to climate chambers with continuous mild heat (CMH: 31°C day, 25°C night; 14h light [-200 µmol m$^{-2}$ s$^{-1}$ at plant height, Philips fluorescent lamps], 10 h dark; 70-80% RH). Two weeks later, pollen from freshly opened flowers was used for assessing viability.

**Figure 1. Schedule for sampling of anthers during and after a 4-day MH treatment.**

Tomato plants were stressed with MH for 4 days (from 13 to 10 DBA). For comparing ROS scavenging gene expression, enzyme activity, lipid peroxidation between control and the 4-day MH, anthers were collected during the last day of MH (i.e. before start of the 33°C period, “MH0”; 6 h into the MH period, “MH6”; at the end of the MH period, “MH12”), and in the middle of subsequent days.

**Gene expression analysis**

A gene set consisting ROS scavenging genes was used for gene set enrichment analysis based on microarray data from control, 1-day and 4-day MH treated samples (see chapter 2 for details). For qRT-PCR, trizol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used to extract total RNA, followed by RNA concentration and quality check with Nanodrop (ThermoScientific, Thermo Fisher Scientific), and DNA digestion with DNase I. Subsequently, cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Primers for genes of interest (Supplementary Table S1) were designed with Beacon Designer software (PREMIER Biosoft International, Palo Alto CA, USA). Primers, SYBRGreen mix (Bio-Rad) and cDNA sample were mixed to a total
volume of 25 µL. A 96-well thermocycler (Bio-Rad iCycler) was used for real-time RT-PCR reactions which followed a two-step protocol: 95°C for 3 min and 40 cycles of 95°C for 15 s, 60°C for 45 s. Subsequently, LingRegPCR software (Ruijter et al., 2009) was used to calculate the average amplification efficiency per primer pair, which was then combined with Cq-values for computation of relative expression levels, which were then normalized against 4 reference genes, CAC, SAND, LeEF1α and RPL8, using GeNorm (Vandesompele et al., 2002).

**Determination of antioxidant enzymes’ activities**

Pools of three anther cones were homogenized in 150 µL of 0.1 M potassium phosphate buffer (pH 7.0) with 1.0 % polyvinylpyrrolidone. The extract was centrifuged at 18000 g for 10 min. The supernatant was stored at −80°C before use. Protein concentration was determined as described by Bradford (1976) using bovine serum albumin as a standard. CAT (EC 1.11.1.6) activity was determined by using the Amplex®Red Catalase Assay Kit (Molecular Probes) according the manufacturer’s instructions. Assays were performed with 5 µL protein extract and values were corrected for protein content to give arelative activity. APX (EC 1.11.1.11) activity was determined as described by Nakano and Asada (1981) with minor modifications. A reaction mixture containing 30 µg of protein, 0.5 mL 1 mM ascorbate and 0.5 mL 0.5 mM H\textsubscript{2}O\textsubscript{2} was prepared, and absorbance at 290 nm was determined after 30 and 240 seconds and the difference was used for calculation of relative activity.

**Lipid peroxidation measurement**

The level of lipid peroxidation was evaluated by determining malondialdehyde (MDA) accumulation, with the thiobarbituric acid reactive substances assay (Jambunathan, 2010). Samples were ground into fine powder and homogenized with 1 mL 80% ethanol. After centrifugation at 12000 rpm for 10 min, 300 µL supernatant was mixed with an equal volume 0.5% (w/v) 2-thiobarbituric acid in 20% trichloroacetic acid. Samples were incubated at 95°C for 30 min. After cooling and centrifugation at 12000 rpm for 5 min, the absorbance of the resulted supernatant was measured at 532 nm and 600 nm using a plate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA). A calibration curve was generated by using 1,1,3,3-Tetraethoxypropane (T9889; Sigma-Aldrich) as a standard.

**Pollen viability evaluation**

In melatonin sprayed Micro-Tom plants, pollen viability was assessed by means of impedance flow cytometry with AMPHA Z30 (Amphasys AG, Lucerne, Switzerland). The manufacturer’s instructions were followed and 3 freshly opened flowers per plant (n=5
plants) were pooled and analysed at day 7 and 8 after the 4-day MH treatment.

In experiment with the AtGRXS17 ectopic expression tomato lines, pollen viability was assessed by an in-vitro pollen germination assay. In brief, collected anther cones were cut into 4 pieces and hydrated for 30 min under humid condition. Later on, pollen was incubated in 0.5 mL artificial germination medium (25% (w/v) PEG 4000, 5% (w/v) sucrose, 1 mM KNO₃, 1 mM Ca(NO₃)₂•4H₂O, 1.6 mM H₃BO₃, 0.8 mM MgSO₄•7H₂O) for 1.5 h under constant rotation. The pollen with tubes longer than their diameter was rated as germinated and about 100 pollen were counted for germination rate. Per genotype, 30 flowers from 3 plants were analysed.

Statistical analysis
All data, except for pollen viability, were log-transformed in order to correct for heterogeneity of variance. Data on ROS gene expression, CAT and APX activity and lipid peroxidation were analysed by two-way ANOVA, taking temperature treatment and time point of sampling as main factors. If temperature treatment or the interaction between the two main factors was significant (P≤0.05), Student’s t-test was applied to compare the difference between control and 4-day MH at the various time points separately. Data on pollen viability from the melatonin spray experiment were analysed by Student’s t-test to show whether melatonin spray has an effect or not. Data on gene expression from the melatonin spray experiment were analysed by one-way ANOVA and means of treatments were separated by Tukey’s Post-Hoc test. Pollen viability for the experiments with ectopically expressed AtGRXS17 in tomato was analysed by one-way ANOVA and LSD was employed to compare transgenic lines with wild type. All statistical analysis were performed with SPSS v20 (IBM, NY, USA).

Results
Expression of ROS scavengers during and following MH stress
We previously showed that 4 days of MH during the meiosis to early microspore stage of pollen development severely reduced viability of the resulting mature pollen (Figure 2C, D from chapter 2) and performed a transcriptome analysis on anthers that received either a control treatment or 1 or 4 days of MH (Figure 7A from chapter 2). As a proxy for enhanced ROS levels, we queried this data for up-regulation of a broad set of ROS-scavenging-related genes using gene set enrichment analysis and found that it was marginally significantly enriched on the day after either MH treatment (Figure 2A).
Chapter 3

Figure 2. Transcription analysis of ROS scavenging genes upon MH treatment.
A) Gene set enrichment analysis of ROS scavengers among 3 comparisons, 1d versus CT, 4d versus CT and 4d versus 1d (1d: 1-day MH, 4d: 4-day MH, CT: control; see chapter 2 for details). The blue from colour key represents down regulation, while red for up regulation. B) Comparing expression of 15 ROS scavenging genes between control and 4-day MH at 9 time points (see Figure 1). The significance level of main effects from temperature and time point and their interaction were, respectively, indicated for each gene in the form of temperature/time points/interaction. At each time point, significant differences between control and 4-day MH were labelled with stars. *, significant effect (two-way ANOVA) or significantly different between control and MH treatment (Student’s t-test), P≤0.05; **, P≤0.01; ***, P≤0.001. n.s., not significant. Values were the mean ± SE.
To better understand the transcriptional response of ROS scavenging genes during 4-day MH and subsequent anther development, the expression of 15 genes were quantified by real-time qRT-PCR at 9 time points in flower buds that received the last day of MH treatment at 10 DBA. Many of the genes turned out to be developmentally regulated (Figure 2B). Nine genes, namely CAT1, CAT3, APX3, APX4, copper-zinc superoxide dismutase 3 (CSD3), manganese superoxide dismutase 1 (MSD1), glutathione reductase 1 (GR1), glutathione peroxidase (GPX4 and GPX8), maintained a relatively low transcript level during early pollen developmental stages, but their expression was highly induced at late stages, on 3 or 1 DBA. The reverse pattern was observed for APX2 and APX5 which showed high gene expression level at the early pollen developmental phase. Expression of the rest of the genes showed a less distinct pattern over the course of pollen development (Figure 2B). Most of the investigated genes showed a very similar transcript level in the 4-day MH and control treatments, or only sporadic deviations. Two genes, CAT1 and APX3 showed a significantly higher expression in the samples taken during the last of the 4 days of MH, i.e. at 10 DBA, but not afterwards (Figure 2B).

![Figure 3. Comparison of APXT and CAT activity between control and 4-day MH treatment.](image)

**Figure 3.** Comparison of APXT and CAT activity between control and 4-day MH treatment.

The enzyme activity was determined at 9 time points (explained in Figure 1) for A) APX activity and B) CAT activity. The significance level of main effects from temperature and time point and their interaction were, respectively, indicated for each enzyme in the form of temperature/time points/interaction. At each time point, significant differences between control and 4-day MH were labelled with stars. *, significant effect (two-way ANOVA) or significantly different between control and MH treatment (Student’s t-test), P<0.05; **, P<0.01; ***, P<0.001. n.s., not significant. Values were the mean ± SE.

**ROS scavenger activity**

Because effects of MH on APX and CAT gene expression were observed, we tested the activity of these two enzymes, during the last day of the 4-day MH treatment and on subsequent days (Figure 3). APX activity was relatively stable over time, not reflecting the developmental gene expression pattern of a specific APX gene (Figure 2B). Overall,
4-day MH increased APX activity, but when looking at days separately, a significant difference was found for 6 DBA, only (Figure 3A). CAT activity was high at 10-8 DBA and dropped on 6 DBA, somewhat reflecting the expression pattern of CAT3, but without clear upregulation at the last stage (Figure 2B). In the 4-day MH treatment, CAT activity was enhanced in the morning of the last treatment day (10 DBA, MH 0h), but not at later time points (Figure 3B). Thus, ROS scavenger activity was only mildly effected by MH.

**Lipid peroxidation**

When ROS level becomes excessive, peroxidation of lipids occurs, giving rise to the formation of MDA, which is widely used as a physiological parameter to examine the damage of ROS on lipids. Under control conditions, MDA content was relatively low and stable during early pollen development, whereas it peaked at 5 DBA, corresponding to late uni-cellular microspore and mitosis stage. Afterwards, it returned to a level comparable with the earlier developmental phase. Comparison between control and treatment showed that 4-day MH did not significantly influence lipid peroxidation (Figure 4).

![Figure 4. Analysis of lipid peroxidation from control and 4-day MH conditions.](Image)

Lipid peroxidation, as revealed by MDA accumulation, was compared between control and 4-day MH at 9 time points (explained in Figure 1). The significance level of main effects from temperature and time point and their interaction were, respectively, indicated in the form temperature/time points/interaction. ***, significant effect (two-way ANOVA), P≤0.001. n.s., not significant. Values were the mean ± SE. FW: fresh weight.

**The effect of melatonin and ectopic expression of glutaredoxin on pollen thermotolerance**

If ROS levels and ROS damage limit pollen development, relief of these factors should improve pollen thermotolerance. Melatonin is an antioxidant that directly detoxifies ROS molecules, like hydroxyl radical and H$_2$O$_2$. Exogenous application of melatonin resulted
in a slight, but significant increase in pollen viability when plants were sprayed with 100 µM and 200 µM melatonin 1 or 24 h before the start of the 4-day MH treatment (Figure 5A).

As components of ROS network, GRX, belonging to the thiolredoxin family, can reversibly reduce the disulphide bonds from substrate proteins, thereby maintaining cellular redox status (Rouhier et al., 2008). Ectopic expression of *AtGRXS17* in tomato (Wu et al., 2012) significantly enhanced pollen viability under CMH condition during flowering (Figure 5B).

**Figure 5. The effect of increasing antioxidant capacity and reducing ROS damage to proteins on pollen performance.**

A) Three concentrations of melatonin was sprayed 1 or 24 h before 4-day MH treatment and pollen viability was determined with freshly open flowers at 7 and 8 days after the 4-day MH treatment. Pollen viability of melatonin sprayed plants was significantly (Student’s *t* test, **, *P*≤0.01) higher than that of plans without spray. B) Pollen phenotypic of *AtGRXs17* ectopic expression tomato lines. Wild type and transgenic lines were subjected to continuous mild heat conditions and flowers completely developed during heat were used for pollen viability determination. *, significantly different between WT and transgenic lines (one-way ANOVA with LSD), *P*≤0.05; ***, *P*≤0.01. Values were the mean ± SE. WT: wild type.

**Discussion**

**Accumulation of ROS under heat conditions**

ROS have unequivocally been shown to be involved in heat induced damage of vegetative cells (Ceylan et al., 2013a; Du et al., 2013). ROS levels in anthers were shown to peak during tapetum degeneration in Arabidopsis and rice (Hu et al., 2011; Xie et al., 2014; Yi et al., 2016) and failure of ROS scavenging led to tapetal dysfunction and pollen abortion in rice (Hu et al., 2011; Luo et al., 2013). However, to answer whether excessive ROS levels are also involved in heat-induced pollen sterility, direct measurement and localization of ROS molecules are required. We tried various chemical assays, e.g. nitroblue tetrazolium
for superoxide (Fryer et al., 2002), Diaminobenzidine tetrahydrochloride and potassium iodide for hydrogen peroxide (Fryer et al., 2002; Velikova and Loreto, 2005) and 2',7'-dichlorodihydrofluorescein diacetate for total ROS (Sandalia et al., 2008) to examine ROS levels, but obtained highly variable results, high background from wounding or insensitive staining in mature pollen only (data not shown). This precluded testing the effect of MH during anther or pollen development. Diaminobenzidine tetrahydrochloride staining was used before to study effect of heat on rice anthers (Bagha, 2014), but here, the temperature treatment was given during the assay incubation, which means that the comparison with control is not valid. In wheat, however, hydrogen peroxide was detected in pre-anthesis stage pollen using the KI method, and the level was reported to increase upon a short heat shock of 42°C (Kumar et al., 2014).

Instead of measuring ROS levels, we determined the extent of lipid peroxidation, which is a general type of damage upon enhanced oxidative capacity. In contrast to accumulation of MDA upon heat in vegetative tissue (Ceylan et al., 2013b; Du et al., 2013), the 4-day MH from 13 to 10 DBA did not enhance overall anther MDA levels, implying that there is no large, general increase in oxidative stress in the whole anther during and after the 4-day MH. Thus, if damage by ROS plays a role in pollen failure, it is likely that this occurs in a small subset of anther tissues only.

Indirect evidence for ROS accumulation under heat

As an alternative to direct quantification of ROS and oxidative damage, we measured ROS scavenger gene expression and enzyme activity, which are regulated by ROS levels in a feed forward fashion (Mittler, 2002). Among those investigated scavenger genes, five were induced by the 4-day MH treatment over the time series as a whole, and in particular, CAT1 and APX3 were upregulated at 10 DBA, that was during the last day of the 4-day MH. Only one gene, GR2, showed reduced expression. A short-term heat shock also enhanced the expression of tomato APX3 (Frank et al., 2009) and its Arabidopsis orthologue, AtAPX2 (Suzuki et al., 2013b), indicating a common response to MH and strong heat shock. APX enzyme activity was up-regulated over the whole experiment, like APX1 and APX3 gene expression, but not showing the up-regulation of APX3 at 1 DBA. Similarly, CAT activity was upregulated at the earliest time point, like CAT1 gene expression, but did not follow the pattern of this gene after that. Instead, its pattern was more alike that of CAT3 gene expression, with a drop at 8-6 DBA, before pollen mitosis, but again without the up-regulation at 1 DBA. A proteomic study that detected two of the proteins, CAT3 and GR1, in developing pollen, however, found a pattern similar to the corresponding transcripts in this study (Chaturvedi et al., 2013). The apparent deviation between scavenger enzyme expression and activity may well be due to an additional, post-transcriptional layer of
regulation (Begara-Morales et al., 2016).

An important question is, to what extent the observed gene expression and activity profiles reflect endogenous ROS levels. Based on the indications that under normal condition, ROS levels are high during tapetum degeneration and at pollen maturity (Hu et al., 2011; Xie et al., 2014; Yi et al., 2016), none of the scavengers seem to be reliable markers for the whole anther at all developmental stages. Possibly, their profiles include both a developmental component and tissue specificity. This leads to the hypothesis that APX2 and APX5 are preferentially expressed in the tapetum, APX3, APX4, CAT1, CSD3, MSD1 and GPX4 in pollen, and GRI, GPX8 and CAT3 in both cell types. The higher expression of APX3, CAT1, GPX4 and GRI upon the 4-day MH would then suggest an enhanced ROS response in pollen (Figure 2B). Unfortunately, there is no data contrasting gene expression of developing pollen and surrounding sporophytic tapetal or anther tissue to confirm this. Adding to the complexity, however, there is extensive interaction between heat and ROS signalling pathways (Driedonks et al., 2015). In particular, APX genes were responsive to heat in a ROS-independent fashion, due to the presence of heat shock elements in their promoters and the promoter of another main regulator, ZAT12 (Rizhsky et al., 2004; Schramm et al., 2006; Storozhenko et al., 1998).

Taken together, the enhanced expression and activity of several ROS scavengers may reflect enhanced ROS levels.

**ROS levels and ROS damage may limit pollen development**

Exogenous application of melatonin, which can act as a radical scavenger (Reiter et al., 2015), resulted in higher pollen viability after the 4-day MH treatment, and similar results were obtained in rice after spray with other antioxidants (Fahad et al., 2016), suggesting that oxidative stress is a limiting factor for pollen development under MH circumstances. However, the physiological effects of melatonin treatment are not well known. Indeed, melatonin pre-treatment led to higher expression of heat responsive HSF A2 and HSP70 genes, and of the ROS scavenger CSD1 at 1 h after start of the 4-day MH treatment (Supplementary Figure S1). Thus, whether melatonin acts through its antioxidant activity or as a priming agent is not clear.

Better pollen tolerance to CMH was found in AtGRXS17 overexpressing tomato lines, which were previously reported to be more heat tolerant in vegetative tissue as well (Wu et al., 2012). The enhanced vegetative thermotolerance was associated with higher ROS-removal ability.

**Conclusion**

Taken together, the transcriptional and enzymatic activity response of ROS scavengers and
improved pollen performance by modifying ROS scavenging and damage hint towards a role for ROS in tomato pollen heat tolerance. However, further investigation is required; in particular, stronger proof of ROS accumulation upon high temperature is necessary. It is likely that not all cells in the anther are equally sensitive to MH, because normal vegetative growth and development are not strongly affected by such a temperature regime. Thus, future research efforts need to focus on tissue and cell level analysis. A good option could be to use genetically encoded modified fluorescent protein probes which can sense cellular redox status to provide non-invasive and possibly even real-time data on ROS kinetics at cell and tissue level (Costa et al., 2010; Fujikawa et al., 2016; Lukyanov and Belousov, 2014; Meyer and Brach, 2009).
The involvement of reactive oxygen species in tomato pollen heat tolerance

References


The involvement of reactive oxygen species in tomato pollen heat tolerance


## Supplementary materials

Table S1. Primer sequences used in this study.

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Table S1. Primer sequences used in this study. (continued)

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Figure S1. Effect of melatonin pre-treatment on gene expression.

Melatonin (0, 100 and 200 µM) was sprayed 1 h before start of the 4-day MH treatment and samples were taken at 1 h into the heat treatment. Control samples did not receive melatonin and heat. Relative gene expression was measured by using qRT-PCR. Values were the mean ± SE. Treatments differ significantly from each other were labelled with different characters, P≤0.05.
High temperature affects vegetative and reproductive trait performances and trait correlations in tomato (*Solanum lycopersicum*)

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Abstract

Long-term mildly high temperatures have adverse effects on plant vegetative and reproductive development and reduce crop yield. To better understand the importance of male and female fertility for tomato fruit set ability under such conditions and test whether heat tolerance levels among and between reproductive and vegetative traits of genotypes correlate with each other, tomato cultivars were subjected to heat stress to evaluate variation and trait correlations in heat tolerance levels. The continuous mild heat (CMH) caused significant decreases in performance of nearly all studied traits, i.e. pollen viability, pollen number, female fertility, fruit set, flowers per inflorescence, but not in inflorescence number. In addition, the traits varied between genotypes, under control and CMH conditions. Nagcarlang, Saladette and Malintka 101 produced a relative higher percentage of viable pollen under CMH. For fruit set under heat, only cultivars that have been previously reported as being heat-tolerant produced fruits with seeds. Correlation analysis revealed relationships between traits within a given temperature treatment, but not between them. Under CMH condition, fruit set was positively correlated with pollen viability, as well as flowers per inflorescence. In addition, no strong correlations were found between vegetative and reproductive traits. These data confirm the importance of pollen viability for fertility under high temperature. We suggest that some of the tested genotypes may be suitable for analysing the genetic architecture of reproductive tolerance traits.
Introduction

When plants experience high ambient temperatures, it may lead to disruption of cellular and organismal homeostasis, also known as heat stress. Basic physiological processes, such as photosynthesis, assimilate partitioning, growth and development are adversely affected (Bokszczanin et al., 2013; Wahid et al., 2007). One of the major effects of high temperatures is the limitation of reproductive success, which commonly translates into yield loss in agricultural settings (Asseng et al., 2011). Despite the wide occurrence of this phenomenon among plant species, the underlying mechanisms have not been well understood (Müller and Rieu, 2016; Zinn et al., 2010).

Tomato (*Solanum lycopersicum*) is an important horticultural crop that also functions as a research model for the plant family of Solanaceae. The optimal daily mean temperature for tomato fruit set is between 21-24°C (Geisenberg and Stewart, 1986), but the field cultivation of this crop in subtropical regions inevitably results in plants being exposed to higher day and night temperature for successive days or even weeks during the reproductive growth phase, which can greatly hamper fruit set (Peet et al., 1998, 1997; Sato et al., 2000). Depending on the maximum day and night time temperatures, the frequency and the duration of the exposure, heat has a suite of effects on reproductive development and physiology in tomato. When experiencing pre-anthesis CMH, flowers had reduced pollen production, pollen viability, pollen shedding, ovule viability and stigma receptivity, but increased physical distance between stigma and anther cone (Kinet and Peet, 1997). While all these abnormalities can occur, it is not fully clear which aspects are the most limiting for tomato fruit set under high temperature and whether tolerance levels for the various processes are related to each other. Screening of sets of tomato cultivars and wild relatives for reproduction under high temperature revealed considerable, heritable natural variation in heat tolerance and, although often not all traits were measured, several studies suggested that viability of male and female gametes and the level of style protrusion are major determinants for reproductive success under these conditions, dependent on the cultivars studied (Bhattarai et al., 2016; Dane et al., 1991; Levy et al., 1978; Rick and Dempsey, 1969; Rudich et al., 1977; Saeeed et al., 2007).

Here, we evaluated a variety of traits in a diverse set of tomato cultivars under control and CMH conditions, in order to 1) assess the importance of male and female fertility in determining tomato fruit set under such conditions, 2) test whether heat tolerance levels among and between reproductive and vegetative traits correlate with each other, and 3) identify genotypes suitable for studying the genetic and physiological differences underlying variation in reproductive heat tolerance.
Materials and methods

Plant material
Thirteen inbred cultivars of tomato (*Solanum lycopersicum*) were obtained from various sources (Table S1). Five of them were explicitly reported to be heat tolerant regarding fruit set, while for eight cultivars no information with respect to heat tolerance was available (Table S1). Seeds were sown in standard potting compost (Lentse Potgrond number 4, Horticoop B.V., Katwijk, The Netherlands) and covered with vermiculite. Ten days later, seedlings were transferred into separate pots and after 20 days seedlings were transplanted into 12-liter pots filled with the same potting compost supplemented with slow-release fertilizer (4 g L\(^{-1}\) Osmocote Exact Standard 3-4 M, Evris International B.V., Geldermalsen, The Netherlands). Plants were grown under standard greenhouse conditions with 16-hour light period (supplemented with artificial light from 600W sodium lamps if natural light intensity fell below 250 µmol m\(^{-2}\) s\(^{-1}\)) and temperature of about 25°C in the day (minimum set to 20°C) and 19°C in the night (minimum set to 17°C).

Plant phenotyping
When the first inflorescences were detectable by eye, all inflorescences were removed and plants were transferred to climate chambers with CMH (32°C/26°C, day/night; 14h light with intensity of ~200 µmol m\(^{-2}\) s\(^{-1}\) at plant height; 70-80% RH) or control conditions (25°C/19°C, day/night). Two weeks later, pollen viability (PV) and pollen number (PN) were evaluated on newly formed flowers as described by Rodriguez-Riano and Dafni (2000). In brief, anther cones were cut into 4 pieces and pollen were released into staining buffer consisting of peroxidase indicator (Sigma 3901-10VL), 200 µL 3% H\(_2\)O\(_2\), and 50 mL 10 times diluted Trizmal buffer (903C; Sigma-Aldrich, St Louis, MO, USA) by vortexing. The resulting pollen suspension was incubated for 20 min at 37°C and loaded onto a haemocytometer. Dark stained pollen was considered as viable. Per flower approximately 100 pollen were assessed for PV analysis. The number of pollen in 25 squares (0.1 µL) of the haemocytometer was counted and converted to PN based on total resuspension volume. In addition, inflorescence number (IN; i.e. from the whole plant) and flowers per inflorescence (FPI; n=3 inflorescences per plant) were recorded. To determine fruit set (FS), five flowers per plant were tagged and mechanically-selfed and kept in CMH or control conditions for one more week before transfer back to the normal greenhouse. In addition, immediately after relocation to the greenhouse, five flowers per plant were pollinated with pollen from control treatment to determine female fertility (FF). Per cultivar and treatment 2 to 5 plants were analysed.
Seedling survival assay

Seeds were sown in trays with soil as described above. In order to obtain seedlings of similar developmental stage, the exact sowing date differed among tomato cultivars. After sowing, the trays were kept in a growth cabinet (25°C/19°C, day/night; 12h light with intensity of ~250 µmol m$^{-2}$ s$^{-1}$ at plant level supplied by Philips Green Power LED DR/B/FR 120 lamps, 12h dark; 60% RH) for 12 days. Uniformly developed seedlings with the first and second true leaf visible were subjected to a 50°C heat treatment for 6 h, in the dark. Afterwards, seedlings were moved to control to allow recovery for 1 week. Seedlings with shrunken and dying stem beneath the apical meristem were considered as dead and therefore, seedling survival rate (SR) was calculated. The whole experiment was repeated three times (n=19-62 seedlings per cultivar per time).

Ion leakage assay

The membrane stability of tomato leaves was determined by ion leakage assay. Seedlings were grown as described above. Before emergence of the 1st truss, 9 leaf discs were collected from the 3rd to 5th fully developed leaf (counted from the top to the bottom). Leaf discs were washed with deionized water 3 times for 5 minutes per time. Cleaned leaf samples were transferred into 50 mL tubes filled with 15 ml deionized water and incubated at 42°C for 3 h. For each tube conductivity was measured immediately after cooling down to room temperature (“E1”), 1 h later (“E2”) and again after disrupting the leaf cells by the incubation at 100°C for 1 h (“E3”). Ion leakage (IL) was calculated as the average of E1/E3 and E2/E3. The experiment was repeated three times (n=3 plants per cultivar per time).

Statistical analysis

PV, SR, FS and IL data were logit transformed [value'=$\ln((value+1)/(101-value))$] and PN and FF data were log transformed [value'=$10^{\log(value+1)}$] before analysis to improve the normality and reduce heteroscedasticity of the data. The overall effects of treatment and cultivar were analysed by means of two-way ANOVA with heat treatment and cultivar as fixed factors since we were also interested in the differences among the 13 tomato cultivars. The interaction between the two main effects indicated whether cultivars responded differently to treatments. In order to establish which cultivars differed from each other, one-way ANOVA followed by Tukey’s Post-Hoc test was performed for each trait and each temperature treatment separately. In addition, the response to temperature regime was tested for each cultivar separately by means of Student’s t-test. All statistical analyses were based on mean value of each plant. To examine whether heat influenced variance proportions explained by various components, lowest level data were separated in heat and control groups and used to calculate sum of squares from each factor with a
nested ANOVA design. Sum of squares of each component were divided by total sum of squares to calculate explained variance. To explore relationships among traits, Pearson correlations were calculated by using the mean value of cultivars with the Hmisc package (Frank and Harrell, 2015). Clusters were generated based on correlations with P≤0.05 and were graphically represented using the igraph package (Csardi and Nepusz, 2006) in the software environment R (R Core Team, 2015). Without explicit mention, all other statistical analysis were done with SPSS v.20 (IBM, NY, USA).

Results

Trait variation in a set of tomato cultivars

To describe natural variation for tolerance under CMH condition and detect correlations between traits, we analysed a number of traits in 13 tomato cultivars (genotypes), including 5 genotypes that were previously described as being heat-tolerant regarding reproductive performance (Table S1). To determine the contributions of genetic and environmental factors to the phenotype, the proportions of phenotypic variance that could be explained by cultivar and plant effects were calculated for control and heat conditions separately (Figure 1). This revealed variable levels of genetic contribution, ranging from ~10% for PN in both conditions to ~50% for FF in CMH. Heat increased the genetic contribution to variation (i.e. differentiation among cultivars) in FF, but did not have such influence on other traits.

Male fertility

To assess male reproductive performance, PV and PN at flower anthesis were analysed under control and CMH conditions. Overall, PV was significantly decreased by CMH treatment, and cultivar differences were found both, in control and CMH (Table 1). There was a significant interaction between cultivar and treatment (Table 1), indicating that not all cultivars responded similarly to the heat. Under control, PV ranged from 47% to 83% and under CMH from 3.9% to 31%, with the cultivars Nagcarlang, Malintka 101 and Saladette showing relatively higher PV under heat condition compared to the other cultivars (Table 1).

Less variation among cultivars was observed for PN (Table 1; also see Figure 1). All 13 cultivars had similar PN under control condition. As for CMH, most of the cultivars produced fewer pollen, except for cultivars NCHS-1 and Hotset, explaining the significant interaction between cultivar and treatment for this trait (Table 1).
High temperature affects vegetative and reproductive trait performances and trait correlations in tomato

Figure 1. Proportions of total trait variances explained by different components. Plants were phenotyped under control temperature (CT) or high temperature (HT, for PV, PN, FF, FS, IN and FPI, the heat is continuous mild heat). Plant explained variance were only calculated for PV, PN and FF. Trait abbreviations: PV, pollen viability; PN, pollen number; FF, female fertility; FS, fruit set; IN, inflorescence number; FPI, flowers per inflorescence; IL, ion leakage; SR, seedling survival rate.

**Female fertility**

FF was determined by evaluating seed set upon manual pollination of freshly opened flowers, with pollen that developed under control, as well as pollination and fruit set taking place under control. Genotypic variation was observed for both temperature conditions, but particularly for CMH (Table 1; also see Figure 1). An overall significant negative effect of CMH on FF was found; at cultivar level the reduction in FF in response to heat was significant for Malintka 101, Hotset, Micro-Tom and Pull (Table 1). Hotset, Nagcarlang and F1 Ninja were the three cultivars that maintained highest FF under CMH condition (Table 1).

**Fruit set ability**

FS, as indicated by the percentage of seeded fruits from 5 tagged flowers upon mechanical self-pollination, was assessed in a subset of cultivars. No cultivar differences in FS were detected under control condition. The CMH treatment drastically reduced FS, with only some cultivars producing seeded fruits (Table 1). Thus, there was a very significant negative effect of temperature treatment. In addition, a marginally significant cultivar effect under CMH, and a significant interaction between cultivar and temperature treatment (Table 1).
Chapter 4

Flowering behaviour

Two flowering-related developmental traits, IN and FPI were recorded under both control and CMH conditions. Overall, IN was not affected by CMH (Table 1). A significant cultivar difference was only found in CMH, with Rubicon having a higher IN than NCHS-1.

On average, CMH decreased FPI by 28% (Table 1). FPI differed significantly among cultivars under both growing conditions, but no difference in response was found among cultivars (Table 1).

Vegetative performance

In addition to focusing on reproductive stage, heat-tolerance of seedlings and membrane stability of leaf cells at vegetative stage was determined. As seedling growth was not visibly affected by CMH, seedling heat tolerance was assessed as survival after exposure to 50°C for 6 h. While SR was 100% under control for all cultivars, the heat treatment reduced it significantly. Differences among cultivars were evident, ranging from 12% to 96%, with Micro-Tom performing best (Table 1).

IL of leaf tissue was used as a proxy for membrane integrity, where higher leakage represents less membrane integrity. Again, as CMH did not affect IL substantially, we used a single high temperature incubation, at 42°C for 3 h, and found significant differences in IL among cultivars (Table 1). The cultivars, M82, Malintka 101 and Pull kept relatively high membrane integrity upon the heat treatment, while Micro-Tom had the lowest (Table 1).

Correlations between traits

To determine relationships among those aforementioned traits, measurements from the different cultivars under both temperature treatments were subjected to Pearson correlation analysis (Table S2) and results plotted as a correlation network (Figure 2). This analysis generated two separate significant clusters. Under control, PV and FF were positively correlated with each other, while FF was negatively correlated with IN. Under the CMH treatment, FS was positively correlated with PV and FPI. Also in this cluster, FPI from control condition was negatively correlated with the level of IL under CMH. FF at CMH did not associate with any other traits. Pearson correlation among treatment responses and traits’ performance from control did not reveal any relevant links (data not shown).
Table 1. Tomato performance under control and heat conditions.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>PV (CT)</th>
<th>PV (HT)</th>
<th>PN(^1) (CT)</th>
<th>PN(^1) (HT)</th>
<th>FF (CT)</th>
<th>FF (HT)</th>
<th>FS (CT)</th>
<th>FS (HT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nagcarlang</td>
<td>76.8 ± 1.5, b(^2)</td>
<td>31 ± 2.3, c ***</td>
<td>66.1 ± 5.9, a</td>
<td>32.2 ± 3.4, ab ***</td>
<td>106.9 ± 28.1, ab</td>
<td>85 ± 4.4, e ns</td>
<td>100 ± 0, a</td>
<td>46.7 ± 16.9, ab *</td>
</tr>
<tr>
<td>Saladette</td>
<td>82.9 ± 0.8, c</td>
<td>16.8 ± 3.6, abc ***</td>
<td>82.6 ± 26.8, a</td>
<td>20.1 ± 4.6, a *</td>
<td>99 ± 1.3, ab</td>
<td>30.7 ± 9.6, bcd ns</td>
<td>100 ± 0, a</td>
<td>44 ± 17.2, a ns</td>
</tr>
<tr>
<td>Malinka 101</td>
<td>69 ± 6.2, abc</td>
<td>20.1 ± 2.4, bc ***</td>
<td>77.3 ± 9.6, a</td>
<td>34.2 ± 6, ab ***</td>
<td>147.1 ± 5.3, b</td>
<td>66.3 ± 7.5, cde *</td>
<td>75 ± 18.9, a</td>
<td>27.5 ± 9.2, a *</td>
</tr>
<tr>
<td>Hotset</td>
<td>68.9 ± 5.2, abc</td>
<td>8.1 ± 2, ab ***</td>
<td>67.8 ± 10.7, a</td>
<td>35.4 ± 8.4, ab ns</td>
<td>162.6 ± 16.3, b</td>
<td>85.3 ± 15.5, de *</td>
<td>100 ± 0, a</td>
<td>6.7 ± 6.7, b ***</td>
</tr>
<tr>
<td>NC HS-1</td>
<td>82.2 ± 4.5, c</td>
<td>4.2 ± 2.2, a **</td>
<td>67 ± 20.7, a</td>
<td>161, 161, 161</td>
<td>11.5 ± 11.5, a, n.d.</td>
<td>80, 26.7 ± 26.7, ab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M82</td>
<td>70.7 ± 4.5, bc</td>
<td>3.9 ± 1.1,  a ***</td>
<td>99.4 ± 14.8, a</td>
<td>45.7 ± 5.9, ab **</td>
<td>63.4 ± 9.8, ab</td>
<td>52.2 ± 10.6, cde ns</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Micro-Tom</td>
<td>69.7 ± 2.7, abc</td>
<td>6.9 ± 1.8, ab ***</td>
<td>77.8 ± 8.1, a</td>
<td>29.3 ± 8.3, ab **</td>
<td>44.1 ± 5.9, ab</td>
<td>13.4 ± 16, ab ***</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Moneyberg</td>
<td>72.2 ± 2.3, bc</td>
<td>8.8 ± 2.3, ab ***</td>
<td>101.7 ± 8.8, a</td>
<td>22.1 ± 3.1, a ***</td>
<td>89.8 ± 14.8, ab</td>
<td>46.9 ± 10.5, bcde ns</td>
<td>96 ± 4, a</td>
<td>0 ± 0, ab ***</td>
</tr>
<tr>
<td>Ninja</td>
<td>46.7 ± 5.8, a</td>
<td>6.1 ± 2, ab ***</td>
<td>86.6 ± 12.4, a</td>
<td>33.8 ± 4.2, ab **</td>
<td>41.7 ± 23.9, a</td>
<td>80.2 ± 8.7, de ns</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Pull</td>
<td>61.3 ± 2.4, abc</td>
<td>8.1 ± 3.9, ab ***</td>
<td>97.1 ± 5, a</td>
<td>51.4 ± 9.9, ab *</td>
<td>45.6 ± 4.9, ab</td>
<td>27.1 ± 4.2, bc *</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Red Setter</td>
<td>56.9 ± 7.8, ab</td>
<td>5.4 ± 1.2, ab ***</td>
<td>88.9 ± 9.4, a</td>
<td>41.5 ± 6.2, a **</td>
<td>42.7 ± 10.1, ab</td>
<td>28.2 ± 3.6, bcde ns</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Rubicon</td>
<td>66.3 ± 3.9, abc</td>
<td>5.7 ± 2, ab ***</td>
<td>78.2 ± 8.2, a</td>
<td>19.3 ± 4.8, a ***</td>
<td>79.2 ± 6.4, ab</td>
<td>57 ± 5.5, cde ns</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tof hamlet</td>
<td>77.2 ± 3.6, bc</td>
<td>5.1 ± 1.3, ab ***</td>
<td>88.6 ± 22.1, a</td>
<td>33.6 ± 4.2, ab **</td>
<td>161, 42.8</td>
<td>100 ± 0, a</td>
<td>0 ± 0, b ***</td>
<td></td>
</tr>
</tbody>
</table>

P<0.001 P=0.056 P<0.001 P=0.089

(see footnotes on next page)
### Table 1. Tomato performance under control and heat conditions. (*continued*)

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>IN (CT)</th>
<th>IN (HT)</th>
<th>FPI (CT)</th>
<th>FPI (HT)</th>
<th>IL (HT)</th>
<th>SR (HT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nagcarlang</td>
<td>11.2 ± 2.2, a²</td>
<td>12 ± 2, ab ns³</td>
<td>7 ± 0.4, abc</td>
<td>5.5 ± 0.3, abc **</td>
<td>25.9 ± 1.7, ab</td>
<td>53.4 ± 25.2, ab</td>
</tr>
<tr>
<td>Saladette</td>
<td>5.5 ± 0.5, a</td>
<td>7.3 ± 1.3, ab ns</td>
<td>8 ± 0.5, bc</td>
<td>6.3 ± 1.1, c ns</td>
<td>26.4 ± 2.5, ab</td>
<td>35.2 ± 18.1, ab</td>
</tr>
<tr>
<td>Malinika 101</td>
<td>6.5 ± 0.9, a</td>
<td>6.6 ± 0.6, ab ns</td>
<td>6.4 ± 0.4, abc</td>
<td>4.3 ± 0.3, abc ***</td>
<td>25.2 ± 2, a</td>
<td>21.8 ± 19.4, a</td>
</tr>
<tr>
<td>Hotset</td>
<td>6.3 ± 0.3, a</td>
<td>7.7 ± 1.3, ab ns</td>
<td>6.5 ± 0.5, abc</td>
<td>4.3 ± 0.2, abc *</td>
<td>26.5 ± 2.8, ab</td>
<td>38.9 ± 14.2, ab</td>
</tr>
<tr>
<td>NCHS-1</td>
<td>5 ± 0, a</td>
<td>4.3 ± 1.1, a ns</td>
<td>4.8 ± 0.3, ab</td>
<td>3.5 ± 0.2, ab *</td>
<td>30 ± 2.9, ab</td>
<td>11.8 ± 10, a</td>
</tr>
<tr>
<td>M82</td>
<td>10.3 ± 2.7, a</td>
<td>7.3 ± 0.9, ab ns</td>
<td>7.2 ± 0.9, abc</td>
<td>4.4 ± 0.3, abc **</td>
<td>24.7 ± 2.5, a</td>
<td>37.5 ± 6, ab</td>
</tr>
<tr>
<td>Micro-Tom</td>
<td>13.1 ± 1.4, a</td>
<td>9.7 ± 0.8, ab ns</td>
<td>5.8 ± 0.4, abc</td>
<td>4.6 ± 0.6, ab ns</td>
<td>40.5 ± 2.8, b</td>
<td>96.1 ± 1.9, b</td>
</tr>
<tr>
<td>Moneyberg</td>
<td>5 ± 0.5, a</td>
<td>5.5 ± 1, ab ns</td>
<td>6.3 ± 0.6, abc</td>
<td>4.1 ± 0.4, ab **</td>
<td>28.3 ± 1.9, ab</td>
<td>54.9 ± 16, ab</td>
</tr>
<tr>
<td>F1 Ninja</td>
<td>8.4 ± 1.5, a</td>
<td>8.6 ± 0.5, ab ns</td>
<td>4.5 ± 0.2, a</td>
<td>3.5 ± 0.2, ab *</td>
<td>34.5 ± 3.9, ab</td>
<td>14.4 ± 8.5, a</td>
</tr>
<tr>
<td>Pull</td>
<td>11.7 ± 1.5, a</td>
<td>10.1 ± 1.5, ab ns</td>
<td>8.1 ± 0.5, c</td>
<td>6.1 ± 0.4, bc **</td>
<td>25.7 ± 4.5, a</td>
<td>n.d.</td>
</tr>
<tr>
<td>Red Setter</td>
<td>11.8 ± 2.5, a</td>
<td>5.9 ± 0.7, ab ns</td>
<td>6 ± 0.6, abc</td>
<td>4.4 ± 0.4, ab ns</td>
<td>27.8 ± 2, ab</td>
<td>31.7 ± 12.4, ab</td>
</tr>
<tr>
<td>Rubicon</td>
<td>9.3 ± 1.6, a</td>
<td>15.6 ± 0.9, b **</td>
<td>5.4 ± 0.4, abc</td>
<td>3.6 ± 0.4, ab **</td>
<td>28.4 ± 2.4, ab</td>
<td>14.8 ± 10.2, a</td>
</tr>
<tr>
<td>Tof hamlet</td>
<td>7.5 ± 0.5, a</td>
<td>6 ± 2.1, ab ns</td>
<td>4.5 ± 0.5, a</td>
<td>3.3 ± 0.8, a ns</td>
<td>37.2 ± 4.8, ab</td>
<td>49 ± 13.6, ab</td>
</tr>
</tbody>
</table>

| Cultivar* | P<0.001 | P<0.001 | P=0.006 | P=0.017 |
| Treatment* | P=0.661 | P=0.001 | P=0.905 | P=0.369 |

Abbreviations: PV, pollen viability; PN, pollen number; FF, female fertility; FS, fruit set; IN, inflorescence number; FPI, flowers per inflorescence; IL, ion leakage; SR, seedling survival rate. CT: control; HT: high temperature (for PV, PN, FF, FS, IN and FPI, the heat is continuous mild heat). All the values were the mean ± SE.

²PN values have to be multiplied by a factor 2000.
³Values in every column were analysed by one-way ANOVA and means were separated by Tukey’s Post-Hoc test.
⁴Trait from CT and HT were compared by Student’s t-test, and significance were added in HT trait column, except for SR and IL.
⁵Some values are excluded for one-way ANOVA and Student’s t-test due to insufficient replication. (n.d., not determined).
⁶Two-way ANOVA was done to show the effects of cultivar, treatment and the interaction.
⁷IL was not evaluated at CT, so no treatment effects were determined for the set and individual cultivars.
⁸SR was always 100% in CT (data not shown).
Table 1. Tomato performance under control and heat conditions.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Treatment</th>
<th>Treatment*cultivar</th>
<th>seedling survival rate. CT: control; HT: high temperature (for PV, PN, FF, FS, IN and FPI, the heat is continuous mild heat). All the values were the mean ± SE.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nagcarlang</td>
<td>1</td>
<td></td>
<td>2 ± 0, ab ns 5.5 ± 0.3, abc ** 53.4 ± 25.2, ab</td>
</tr>
<tr>
<td>Saladette</td>
<td>2</td>
<td></td>
<td>7.3 ± 1.3, ab ns 6.3 ± 1, c ns 35.2 ± 18.1, ab</td>
</tr>
<tr>
<td>Malintka 101</td>
<td>3</td>
<td></td>
<td>6.6 ± 0.6, ab ns 4.3 ± 0.3, abc *** 21.8 ± 19.4, a</td>
</tr>
<tr>
<td>Hotset</td>
<td>4</td>
<td></td>
<td>7.7 ± 1.3, ab ns 4.3 ± 0.2, abc * 38.9 ± 14.2, ab</td>
</tr>
<tr>
<td>NCHS-1</td>
<td>5</td>
<td></td>
<td>4.3 ± 1.1, a ns 3.5 ± 0.2, ab * 11.8 ± 10, a</td>
</tr>
<tr>
<td>M82</td>
<td>6</td>
<td></td>
<td>7.3 ± 0.9, ab ns 4.4 ± 0.3, abc ** 37.5 ± 6, ab</td>
</tr>
<tr>
<td>Micro-Tom</td>
<td>7</td>
<td></td>
<td>9.7 ± 0.8, ab ns 4.6 ± 0.6, abc ns 96.1 ± 1.9, b</td>
</tr>
<tr>
<td>Moneyberg</td>
<td>8</td>
<td></td>
<td>5.5 ± 1, ab ns 4.1 ± 0.4, abc ** 54.9 ± 16, ab</td>
</tr>
<tr>
<td>F1 Ninja</td>
<td>9</td>
<td></td>
<td>8.6 ± 0.5, ab ns 3.5 ± 0.2, ab * 14.4 ± 8.5, a</td>
</tr>
<tr>
<td>Pull</td>
<td>10</td>
<td></td>
<td>10.1 ± 1.5, ab ns 6.1 ± 0.4, bc ** n.d.</td>
</tr>
<tr>
<td>Red Setter</td>
<td>11</td>
<td></td>
<td>5.9 ± 0.7, ab ns 4.4 ± 0.4, abc ns 31.7 ± 12.4, ab</td>
</tr>
<tr>
<td>Rubicon</td>
<td>12</td>
<td></td>
<td>15.6 ± 0.9, b ** 3.6 ± 0.4, ab ** 14.8 ± 10.2, a</td>
</tr>
<tr>
<td>Tof hamlet</td>
<td>13</td>
<td></td>
<td>6 ± 2.1, ab ns 3.3 ± 0.8, a ns 49 ± 13.6, ab</td>
</tr>
</tbody>
</table>

Note: PN values have to be multiplied by a factor 2000.

Values in every column were analysed by one-way ANOVA and means were separated by Tukey’s Post-Hoc test.

Trait from CT and HT were compared by Student’s t-test, and significance were added in HT trait column, except for SR and IL.

Some values are excluded for one-way ANOVA and Student’s t-test due to insufficient replication. (n.d., not determined).

Two-way ANOVA was done to show the effects of cultivar, treatment and the interaction.

IL was not evaluated at CT, so no treatment effects were determined for the set and individual cultivars.

SR was always 100% in CT (data not shown).

Discussion

CMH affects reproductive traits, dependent on genotype

At the reproductive stage, CMH had significantly harmful effects on PV, PN and FF, in line with other studies (Firon et al., 2006; Levy et al., 1978; Peet et al., 1998; Pressman et al., 2002; Sato et al., 2006, 2000). The responses of different traits to the heat varied, with an average reduction of 86%, 56% and 39% for PV, PN and FF, respectively. For each of the 13 cultivars, reduction in PV was larger than in FF, which strengthens the notion that development of the male gametophyte is more sensitive to heat than that of the female one (Peet et al., 1998). In addition to the effect of heat, analysis showed large genotypic effects on reproductive traits. In our experiment, three cultivars identified as being heat-tolerant in previous studies, i.e. Nagcarlang, Malintka 101 and Saladette (Abdul-Baki, 1991; Chetelat and Petersen, 2003; Dane et al., 1991; Opeña et al., 1992; Rudich et al., 1977), produced pollen with a relatively high viability when flowers completely developed under CMH. Hotset did not perform well in this respect, but contrasting results have
been reported before for this cultivar (Levy et al., 1978; Dane et al., 1991). Cultivars also
differed for PN, especially its response to the CMH. Here, cultivar NCHS-1 stood out, as
its PN was not affected by temperature and was the highest of all cultivars under CMH
condition. Regarding FF, large variation among cultivars was observed under both control
and CMH temperature regimes. Several cultivars were hardly affected by CMH in this
respect, including Nagcarlang and F1 Ninja, which together with Hotset maintained the
highest FF under CMH condition. Taken together, heat as well as genotype influenced trait
performances, often in interaction with each other.

**PV limits FS under CMH condition**

Living organisms function as physiologically integrated networks, meaning that not
all traits can behave independently. There can be trade-offs, for example due to limited
resources, but also dependencies, such as the requirement for viable pollen to produce
fruits and seeds. In the present study, FS was the most strongly affected trait by the CMH
treatment, which fits with the idea that reproductive success depends on multiple heat-
sensitive sub-processes, leading to a synergistic, stronger effect on final fertility. Because
of the compound nature of FS and potential interactions between sub-traits, it is difficult to
determine the relative contribution of each in the limited set of genotypes used in this study.
For example, although the cultivar Nagcarlang produced pollen with high viability, FS was
likely to be limited by the fact that its style protrudes out of the anther cone (unpublished
data; Dane et al., 1991). This complexity was also reflected by the low heritability of FS
under heat in genetic studies, while the heritability of more simple sub-traits, like style
exertion, was relatively high (Levy et al., 1978). Still, within our set of cultivars we found
a positive correlation under heat between FS and PV, but not with FF. As in other studies
using cultivar sets, the breeding history of the cultivars used here is unknown, meaning that
identified trait correlations may either point at physiological dependencies or similarities
among traits, but could also reflect linkage due to genetic relationships among cultivars.
Similarly, the interpretation of the observed lack of correlation among several traits is
limited by the fact that correlations can only be found if sufficient variation is present in
the genotype set. However, the fact that correlation between PV and FS under CMH has
now been reported multiple times with different sets of tomato cultivars (Akhtar et al.,
2012; Dane et al., 1991; Levy et al., 1978), suggesting that male fertility is indeed a key
determinant for reproductive heat tolerance in this species. This is further corroborated
by two studies in which heat was applied to the male and female parent plants of a cross
separately (Levy et al., 1978; Peet et al., 1998). Similar conclusions were drawn from other
plant species, such as bean, cowpea, groundnut, brachypodium, barley and rice (Ahmed et
al., 1992; Harsant et al., 2013; Prasad et al., 1999; Sakata et al., 2000; Suzuki et al., 2000).

Regarding FF, considerable genotypic variation was present in the cultivar set, especially
under CMH, so the absence of a significant correlation suggests that FF is not limiting FS under the CMH used in the experiment. This corresponds to the relatively low effects of heat on the female side found in reciprocal crosses (Peet et al., 1998), but has not yet been reported in a correlational study with multiple cultivars as performed here. Under control temperature, no positive correlation between either PV or FF and FS was found. This suggests that both male and female fertility are not the main limiting factors for FS under more optimal temperature growth conditions.

**Correlations between vegetative, flowering and reproductive traits**

It was reported that total flower production in tomato was reduced upon experience of severely high temperature conditions (El Ahmadi and Stevens, 1979), but under CMH, flower number seemed not to be affected (Peet et al., 1998, 1997; Sato et al., 2006, 2004). In our cultivar set, CMH did not affect IN, but reduced FPI, the latter of which was also found by Adams et al., (2001). This may suggests that the total flower number is compensated by longer flowering or a higher inflorescence production at later stages. Interestingly, FPI under CMH condition was positively correlated with FS. Similar correlations have also been described by Abdul-Baki (1991), who reported that genotypes that were more heat-tolerant regarding fruit set had more flowers in control and high temperature conditions and also by Kugblenu et al. (2013), who found that under mild heat, cultivars with more FPI showed less flower drop, which likely corresponded to having fewer unfertilised flowers. Although in our data, PV was in the same correlation cluster as FS and FPI, and the two traits correlated significantly with each other, this correlation was not very strong. In a larger study with 38 genotypes, however, FPI clearly correlated with PV (Akhtar et al., 2012).

So far, effects of heat on reproductive and vegetative processes have not often been studied in concert. In the present work, the various cultivars were evaluated for membrane thermo-stability by an in-vitro ion leakage assay and we found that IL belongs to the cluster of correlating traits that includes FS and PV under heat. Also, Camejo et al. (2005) showed that a known heat-tolerant line, Nagcarlang, had more stable membranes than a heat-sensitive. Membrane thermo-stability has often been linked to photosynthetic and respiratory performance under heat (Wahid et al., 2007), but these latter two traits do not seem to be affected by mild heat (Sato et al., 2000), suggesting a different link between membrane and reproductive heat tolerance. By contrast, we did not find any relation between heat tolerance of seedlings and reproductive processes. The finding that Micro-Tom seedlings, with relatively short and thick hypocotyls, were the most heat-tolerant suggests that morphological characteristics can be important at this stage.
Conclusion

Considerable natural variation for reproductive and non-reproductive traits under heat conditions was found. Within the germplasm set under study, PV seemed to be the main factor limiting FS under heat condition. Furthermore, there was considerable evidence that FPI can be used as an indicator of reproductive heat-tolerance in tomato and indication that membrane thermo-stability is also a relevant characteristic when studying the reproductive heat-tolerance. Evaluations at an early developmental stage would make the extended cultivation process that is required to measure reproductive traits unnecessary. However, a larger set of phenotypically variable individuals, e.g. from a segregating population of siblings, would be required to corroborate these findings. Also, the fact that trait correlations were not very strong indicates that the validity of potential markers needs to be confirmed for specific genetic sources used in breeding. Whether there is a physiological basis for the identified correlations or whether the traits have a unique, but genetically linked basis remains to be further investigated. Analysis of the genetic structure behind pollen thermostolerance and the other traits may be pursued using a cross between two contrasting cultivars, such as Nagcarlang and NCHS-1. The suitability of Nagcarlang for such a study is supported by the finding that it was among the best performing genotypes regarding pollen fertility and fruit set under high temperature field conditions and general combining ability of these traits (Bhattarai et al., 2016; Dane et al., 1991).
High temperature affects vegetative and reproductive trait performances and trait correlations in tomato

References

High temperature affects vegetative and reproductive trait performances and trait correlations in tomato

## Supplementary tables

Table S1. Information about cultivars used in present study.

<table>
<thead>
<tr>
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<th>Origin</th>
<th>Reported reproductive heat tolerance</th>
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<td>Nagcarlang</td>
<td>TGRC (LA2661)</td>
<td>Shelby et al., 1978; El Ahmadi and Stevens, 1979; Dane et al., 1991; Bhattarai et al., 2016</td>
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High temperature affects vegetative and reproductive trait performances and trait correlations in tomato.

### Supplementary tables

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**Table S2.** Pearson coefficients ($r$ values) of correlation analysis among physiological traits.

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<th>$FPI$ (HT)</th>
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**Abbreviations:** $PV$, pollen viability; $PN$, pollen number; $IN$, inflorescence number; $FPI$, flowers per inflorescence; $FF$, female fertility; $FS$, fruit set; $IL$, Ion leakage; $SR$, seedling survival rate. CT: control; HT: high temperature (for $PV$, $PN$, $FF$, $FS$, $IN$ and $FPI$, this is continuous mild heat). Significance level of correlations: $^*$, $P\leq0.1$; $^*$, $P\leq0.05$; $^{**}$, $P\leq0.01$; $^{***}$, $P\leq0.001$. 
CHAPTER 5

Mapping quantitative trait loci for heat tolerance at reproductive stage in tomato (Solanum lycopersicum)

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Chapter 5

Abstract

Global warming has become a worldwide concern due to its adverse effects on agricultural output. In particular, long-term mildly high temperatures interfere with sexual reproduction and thus fruit and seed set. Previously, a set of tomato cultivars was evaluated for reproductive performance under such continuous mild heat (CMH) condition and considerable variations was found among cultivars for several key reproductive traits. To uncover the genetic basis of observed variations in tolerance against heat, a bi-parental F$_2$ mapping population was generated from two contrasting cultivars for phenotyping, i.e. pollen viability, pollen number, style length, anther length, style protrusion and flowering characteristics (inflorescence number and flowers per inflorescence). Quantitative trait loci (QTLs) were identified for all these traits, including the one for pollen viability with an LOD score of 17.6, which accounted for 36.3% phenotypic variation. QTLs for some traits were co-localized, indicating trait dependency or potential pleiotropic/broad effect loci. Thus, we conclude that a limited set of major genes determines differences in performance of reproductive traits under CMH condition. The generated material should be useful to identify the underlying genes and their physiological role and we suggest that introgression of specific QTLs into elite tomato cultivars will improve fruit set ability during heat waves in the field.
Introduction

High temperature is one of the major abiotic stress factors affecting plants, having adverse effects on both growth and reproduction. In an agricultural context, this leads to negative effects of heat on yields, as can already be seen from several major crop species (Driedonks et al., 2016; Jha et al., 2014). Temperature projections for the coming century show a continuing increase in global surface temperature and suggest more frequent and more severe heat waves, meaning that crops are even more likely to be exposed to high temperature during their growth period (Pachauri et al., 2014). For instance, the maize cultivation area that suffers from heat was predicted to increase from 15% in 2000 to 44% in 2050 (Gourdji et al., 2013). A better understanding of plant heat tolerance mechanisms and description of existing heat tolerance traits is therefore urgently needed.

A number of basic processes, such as protein folding, maintenance of membrane stability, photosynthesis and assimilate metabolism, are shown to be affected by heat (Bokszczanin et al., 2013; Wahid et al., 2007). When considering long-term mildly high temperatures, representative of heat waves in subtropical climates, life-cycle stages clearly differ from each other regarding sensitivity, with reproductive processes were found to be more vulnerable than vegetative ones (Hall, 1992). This vulnerability applies especially to the meiotic to early microspore stages during pollen development, and 1-3 days after fertilization (Bac-Molenaar et al., 2015; Giorno et al., 2013; Hedhly et al., 2009; Müller and Rieu, 2016). There seemed to be considerable natural variation for reproductive heat tolerance within plant species (Bac-Molenaar et al., 2015; Patel and Hall, 1990; Redona et al., 2009; Shpiler and Blum, 1986) and QTLs for related traits were described (Bac-Molenaar et al., 2015; Esten Mason et al., 2011; Ye et al., 2012). While some of these QTLs were already used in breeding, the underlying genes and their physiological effects were not yet reported.

As an important horticultural crop grown for fruit production, tomato has been extensively studied for heat tolerance at reproductive phase. Similar to other plant species, the reproduction is particularly sensitive to CMH (Kinet and Peet, 1997). Under these conditions, male fertility and the position of the stigma relative to the anther cone seemed to be major factors limiting fruit and seed set (Chapter 4; Dane et al., 1991; Levy et al., 1978). A number of studies assessed tomato cultivars for fruit set under CMH condition and identified relatively well-performing genotypes (Bhattarai et al., 2016; Dane et al., 1991; Levy et al., 1978; Sato et al., 2004, 2000). The largest, multi-year characterizations by the Asian Vegetable Research and Development Center (AVRDC) identified 39 tolerant lines, some of which were reportedly already being utilized in tomato breeding programs (Opeña et al., 1992; Gardner, 2000; Scott et al., 1995). However, to be able to use variation in heat tolerance for fundamental and applied aims, it is necessary to have knowledge
on the genetic basis of the trait. Tomato QTLs associated with reproduction under heat were reported in two studies (Grilli et al., 2007; Lin et al., 2010), but identified markers and linkage groups were not associated with chromosomes, hampering wider use of the findings.

The objective of this study was to dissect the genetic architecture underlying tolerance of key reproductive traits under CMH condition. Tolerance regarding pollen viability was identified in tomato cultivar Nagcarlang, while tolerance with respect to pollen number and stigma protrusion was identified in cultivar NCHS-1 (Chapter 4). Here, in a forward genetic approach, an intraspecific F$_2$ population derived from these two phenotypically contrasting parents was used for QTL mapping.

**Material and methods**

**Plant material**

Tomato (*S. lycopersicum*) cultivars Nagcarlang (LA2661) and NCHS-1 (LA3847) were obtained from TGRC and crossed by using Nagcarlang as mother plant. F$_2$ seeds were collected from a single F$_1$ plant and 180 F$_2$ individuals were phenotyped for QTL analysis.

**Plants husbandry and heat stress phenotyping**

Seeds of the two parental cultivars and the F$_2$ population were sown in standard commercial potting soil (Lentse Potgrond number 4, Horticoop, Katwijk, The Netherlands) with vermiculite scattered on top to cover the seeds. Ten days after sowing, seedlings were transplanted into separate pots with commercial potting soil and at 20 days after sowing, seedling were transferred further to 12-liter pots filled with the same soil supplemented with slow-release fertilizer (4 g L$^{-1}$ Osmocote Exact Standard 3-4 M, Everris International B.V., Geldermalsen, The Netherlands). Plants were grown under standard greenhouse conditions with 16-hour light period (supplemented with artificial light from 600W sodium lamps if natural light intensity fell below 250 µmol m$^{-2}$ s$^{-1}$) and temperature of about 25°C in the day (minimum set to 20°C) and 19°C in the night (minimum set to 17°C).

When the first inflorescences were detectable by eye, all inflorescences were removed and plants were transferred to climate chambers with CMH condition (31°C day, 25°C night; 14 h light, [-200 µmol m$^{-2}$ s$^{-1}$ at plant height, Philips fluorescent lamps], 10 h dark; 70-80% RH). The following phenotypical data were collected: inflorescence number (IN; i.e. number of inflorescences on top 5 branches present 3 weeks after start of CMH), flowers per inflorescence (FPI; i.e. average number of flowers on 3 randomly chosen inflorescences), anther length (AL; i.e. of 6 to 10 newly opened flowers), style length (SL; i.e. of 6 to 10 newly opened flowers), style protrusion (SP = SL – AL; i.e. of 6 to 10 newly opened flowers),
newly opened flowers), pollen viability (PV; i.e. *in-vitro* pollen germination percentage of 6 to 10 newly opened flowers), pollen number (PN; i.e. of 6 to 10 newly opened flowers) and female fertility (FF). To assess PV, mature pollen was hydrated for 30 min and then incubated in 0.5 mL artificial germination medium (25% (w/v) PEG 4000, 5% (w/v) sucrose, 1 mM KNO$_3$, 1 mM Ca(NO$_3$)$_2$$\cdot$4H$_2$O, 1.6 mM H$_3$BO$_3$, 0.8 mM MgSO$_4$$\cdot$7H$_2$O) under constant rotation for 1.5 h. Subsequently, the pollen suspension was loaded on a haemocytometer and pollen with tubes longer than the diameter was rated as germinated; about 100 pollen were evaluated for calculating germination rate. The number of pollen in 25 squares (0.1 µL) of the haemocytometer were counted and values expressed as PN. After phenotype evaluation under CMH, plants were moved back to normal greenhouse conditions and 4 open flowers were immediately pollinated with NCHS-1 pollen from control condition. Seed number of fruits from hand pollination were counted to evaluate FF. During phenotyping, plants were arranged batch by batch (10 plants per batch) due to limited space of climate chambers, and two chambers were used for a higher throughput.

**Genotyping**

Single nucleotide polymorphisms (SNPs) between Nagcarlang and NCHS-1 were identified using the SolCAP SNP array at ENZA Zaden (Enkhuizen, The Netherlands), according to (Sim et al., 2012). 96 SNPs (Table S1) were selected to be relatively evenly distributed over the tomato genome and used for genotyping by KASP assays (LGC, Teddington, UK; Supplemental Table 1) at ENZA Zaden, according to standard procedures.

**Statistical and QTL analysis**

Without explicit mention, all statistical analysis was done with R (R Core Team, 2015). Phenotypic differences between the two parental cultivars were compared by Welch (unequal variance assumption) two samples *t*-test with *t.test* function. In addition, the descriptive statistics of F$_2$ population were calculated and trait frequency distributions were plotted. Pearson correlations between mother plants and cuttings, and among phenotypic traits in the F$_2$ population were calculated and visualized by using Hmisc and igraph package (Csardi and Nepusz, 2006; Frank and Harrell, 2015).

Phenotypic and genotypic data were integrated for QTL mapping with the R/qtl package (Broman et al., 2003). Marker diagnostics was performed and 12 low quality markers were discarded. Furthermore, one marker showing strong segregation distortion (P<0.0001) and two markers highly correlated with segments of different chromosomes were excluded. A genetic map was constructed by *est.map* function with 81 informative markers. Standard interval mapping (SIM) and composite interval mapping (CIM) were applied for QTL detection. For SIM, the whole genome was scanned at steps of 1 cM by *scanone* function...
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with batch and climate chamber added as covariates. CIM was implemented by `cim` function with 1 cM steps and 3 markers were selected as cofactors by forward selection. Default settings were used for all the rest arguments, except for expectation-maximization was chosen as method. Significance thresholds were generated for each trait by determining the LOD values at alpha of 0.05 from 1000 permutations (Churchill and Doerge, 1994). During map construction and QTL analysis, Kosambi map function was employed for the conversion of recombination frequency to genetic distance (Kosambi, 1944). Since similar results were obtained from SIM and CIM, only CIM results were analysed with `fitqtl` to get QTL effects and interactions.

Results

Phenotypic variation under CMH condition

As seed and fruit set under high temperature are expected to be compound, polygenic traits, which reduces the power of correlative analyses, we set out to analyse various sub-traits known to contribute to reproductive success under high temperature, e.g. PV, PN and SP. Performance of the two cultivars previously found to be contrast for these traits under CMH, Nagcarlang and NCHS-1 (Chapter 4), was re-assessed and, again, a larger part of the pollen from Nagcarlang were viable, whereas NCHS-1 produced more pollen, had a shorter style and less style protrusion (Table 1). Additionally, Nagcarlang had higher FF, flowered significantly earlier, produced more inflorescences and formed more flowers per inflorescence under CMH condition than NCHS-1.

In the F$_2$ population, phenotypic data for all traits scattered over wide continuous ranges, indicating quantitative inheritance (Figure 1). FPI, AL and SL followed normal distributions as their kurtosis and skewness values were close to 0. Distribution of the other traits skewed somewhat to the right except for FF (Table 1, Figure 1). Transgressive segregation were observed for AL and FF since the F$_2$ population mean for these two tratis was outside the ranges defined by the two parental population means (Table 1).
Table 1. Phenotype of the two parents and F₂ population under continuous mild heat condition.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Nagcarlang</th>
<th>NCHS-1</th>
<th>F₂ population</th>
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<tbody>
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<td>PV (%)</td>
<td>20 35.6 21 21.1***</td>
<td>180 25.7 11.4 2.7</td>
<td>61.8 0.71 0.51</td>
</tr>
<tr>
<td>PN (x 5000)</td>
<td>20 11.9 21 17.4***</td>
<td>180 13.1 4.3 4.2</td>
<td>28.1 0.73 0.87</td>
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<tr>
<td>FF</td>
<td>14 113.5 9 81.8**</td>
<td>122 119.7 28.3 22.0</td>
<td>185.5 0.51 -0.23</td>
</tr>
<tr>
<td>SP (mm)</td>
<td>20 1.2 21 0.2***</td>
<td>180 1.0 0.7 -0.7</td>
<td>3.3 -0.08 0.32</td>
</tr>
<tr>
<td>SL (mm)</td>
<td>20 7.9 21 6.7***</td>
<td>180 7.9 0.8 5.6</td>
<td>10.1 0.06 -0.02</td>
</tr>
<tr>
<td>AL (mm)</td>
<td>20 6.7 21 6.5</td>
<td>180 6.9 0.5 5.4</td>
<td>8.1 -0.12 0.05</td>
</tr>
<tr>
<td>IN</td>
<td>20 24.8 18 12.2***</td>
<td>180 16.0 3.5 7.0</td>
<td>24.0 -0.64 0.03</td>
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<tr>
<td>FPI</td>
<td>20 5.8 19 4.1***</td>
<td>180 5.2 0.9 2.7</td>
<td>7.7 -0.14 0.01</td>
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</tbody>
</table>

¹Trait abbreviations: PV, pollen viability; PN, pollen number; FF, female fertility; SP, style protrusion; SL, style length; AL, anther length; IN, inflorescence number; FPI, flowers per inflorescence.
²Differences between the means of Nagcarlang and NCHS-1 were compared by Welch’s t-test. **, significantly different from Nagcarlang, P≤0.01; ***, P≤0.001.

Figure 1. Phenotype distribution analysed traits of F₂ population.
A) Inflorescence number (IN); B) Flowers per inflorescence (FPI); C) Style length (SL); D) Anther length (AL); E) Style protrusion (SP); F) Pollen viability (PV); G) Pollen number (PN); H) Female fertility (FF). Mean values of two parents were indicated by arrows. P₁, Nagcarlang; P₂, NCHS-1.
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Reliability of phenotyping method was verified by the comparison of phenotypic data (i.e. PV, PN, SL, AL, SP) between 35 F₂ individuals and cuttings thereof. Strong and positive correlations were found for all these traits, in particular for PV and SP (Figure 2). This indicates that genotypic effects are large relative to environmental effects of the assay, which is crucial for QTL analysis.

Figure 2. Validation of phenotypic data by correlating values from mother plants and cuttings. Correlations were shown as scatter plots, with linear regression in red. Pearson’s r and n values were indicated for each trait. Correlations were significant in all cases (P<0.001). A) Pollen viability (PV); B) Pollen number (PN); C) Style length (SL); D) Anther length (AL); E) Style protrusion (SP).
Figure 3. Trait correlations in the Nagcarlang x NCHS-1 F₂ population.
A) Heat map for Pearson correlations. *, P≤0.05; **, P≤0.01; ***, P≤0.001. B) Significant correlations were picked up for visualization as a network. The width of the edges indicates the strength of correlations, solid and dash lines represent positive and negative correlations, respectively. The colour of edges indicates significance levels, light grey for P≤0.05, grey for P≤0.01, black for P≤0.001. IN, inflorescence number; FPI, flowers per inflorescence; SL, style length; AL, anther length; SP, style protrusion; PV, pollen viability; PN, pollen number; FF, female fertility.

Trait correlations in F₂ population
To assess associations among traits, Pearson correlation analysis was applied (Figure 3A) and significant correlations were used to construct a network (Figure 3B). A positive correlation was found between IN and FPI (Figure 3). As for the size of floral organs, positive correlations were found between SP and SL, and between SL and AL. AL was also positively correlated with PN (Figure 3). In addition, we also observed relatively weak associations. For instance, the negative weak correlation between PV and SL, SP.

Correlation analysis also revealed that FF was an independent trait (Figure 3).

QTL analysis and their interaction
DNA from both parents was hybridized with the SolCAP SNP array to identify polymorphic SNPs. In order to localize the genetic determinants for heat tolerance, DNA samples from F₂ population were genotyped using 96 SNPs that were distributed relatively evenly over the genome. After marker quality filtering, recombination frequencies between 81 markers were used to generate a genetic map covering the 12 chromosomes. QTLs were identified for all the traits, except for FF. Highly similar results were obtained by the use of SIM and CIM (Table 2) and therefore only the results of CIM were used for further evaluation.
### Table 2. QTL overview for all the traits.

<table>
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<th>Closest SNP</th>
<th>LOD</th>
<th>Position</th>
<th>Closest SNP</th>
<th>LOD</th>
<th>a</th>
<th>d</th>
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</table>

1. Trait abbreviations: PV, pollen viability; PN, pollen number; SP, style protrusion; AL, anther length; SL, style length; FPI, flowers per inflorescence; IN, inflorescence number.
2. Position is presented as chromosome@genetic position.
3. a, additive effect (positive value indicates a positive effect from Nagcarlang, negative value indicates a positive effect from NCHS-1); d, dominance effect.
A single, highly significant QTL associated with PV (qPV11) was detected on chromosome 11 (Table 2, Figure S1). qPV11 explained 36.3% phenotypic variation of the trait and additive and dominance effects of the QTL were 9.1 and -3.3, respectively. The allele contributing positively to PV derived from Nagcarlang. The three genotypes at the closest marker differed significantly from each other with respect to PV (Figure 4A). Likewise, a single QTL for PN was identified on chromosome 7 (qPN7) and determined to account for 18.6% phenotypic variation of the trait. The positive effect was contributed by the allele from NCHS-1. The additive and dominance effects were -2.4 and 0.3, respectively (Table 2, Figure S1).

QTLs for SL, AL, SP, IN and FPI were identified as well (Table 2, Figure S1). The two QTLs for SP, on chromosome 1 (qSP1) and 3 (qSP3), co-localised with two for SL (qSL1, qSL3), while a third QTL for SL (qSL2) co-localised with one for AL (qAL2). Of the two additional QTLs for AL, the stronger one (qAL7) co-localised with qPN7 for PN. Two QTLs were found for IN on chromosomes 1 and 8, which interacted epistatically: if qIN1 was homozygous for the NCHS-1 allele, the effect of qIN8 was relatively small, whereas it became larger with qIN1 having one or two Nagcarlang alleles (Figure 4B).

Figure 4. A) Comparison of pollen viability from three groups separated by the genotypes of the closest marker to qPV11. “A”, NCHS-1 allele; “B”, Nagcarlang allele. B) Interaction of two QTLs for inflorescence number (IN). If one of the QTLs is homozygous to NCHS-1, the effect of the other one is small. “A”, NCHS-1 allele; “B”, Nagcarlang allele.
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Discussion

PV and PN
Sufficient viable pollen is required for successful fertilization in flowering plants. The number of viable pollen depends on total pollen number and percentage of viable pollen; both of these were significantly decreased under heat condition. A positive correlation was previously found between tomato PV and fruit set under heat conditions (Levy et al., 1978; Dane et al., 1991; Akhtar et al., 2012) and also our own analysis showed PV was a limiting factor for fruit set under CMH (Chapter 4). In the present study, a single QTL for PV, qPV11, was detected in tomato on chromosome 11, accounting for a relatively large part (36.3%) of phenotypic variation in the population. Still, continuous distribution of phenotypic values indicates that additional undetected small-effect genetic factors must be involved. The QTL position was unique with respect to those for other traits studied here. The only two QTLs for pollen fertility under long-term mild heat described so far, came from a study in rice (Xiao et al., 2011) and these two QTLs had a relatively small effects and explain a comparable portion of phenotypic variance together as tomato qPV11. Taken together, these results indicate that pollen thermotolerance may be determined by relatively few major genes.

A negative effect of heat on PN was reported before (Levy et al., 1978; Firon et al., 2006; Pressman, 2002; Sato and Peet, 2005). Our QTL mapping identified one QTL for PN on chromosome 7, which was also associated with AL, in line with the strong positive correlation between the two traits. The same association was found in wheat, where introduction of a chromosome of a wheat-related species enhanced both anther size and pollen production (Nguyen et al., 2015). It seems likely that a larger anther contains more pollen mother cells and is able to support more developing pollen. Whether the number of pollen produced and the number of pollen released from the anther are independent traits, and which of the two most strongly limits reproduction under heat, is still unclear (Sato et al., 2000; Firon et al., 2006). Future analysis of the effect of qPN7 on fruit setting ability under high temperature should shed light on this.

SP, SL and AL
Inserted stigma is an important trait to ensure self-pollination in cultivated tomato (Chen and Tanksley, 2004; Rick and Dempsey, 1969), while heat leads to SP out of the anther cone. SP depends on the interaction between AL and SL (Chen and Tanksley, 2004). In our mapping population, SP was positively correlated with SL and negatively with AL, indicating both anther and style have effects on the extent of SP. However, the much stronger correlation between SP and SL indicates that an elongated style is the main reason for SP under heat stress. Association among these floral structure traits is also reflected by coincidence of
QTLs. The two QTLs for SP were co-localised with QTLs for SL. By contrast, a QTL on chromosome 2 affected both style and anther length, in the same direction, explaining why it did not contribute to variation in SP. An protruded stigma phenotype is observed often in self-incompatible wild tomato relatives to facilitate outcrossing (Chen and Tanksley, 2004). To reveal how an inserted stigma evolved from the wild ancestors, genetic analysis identified several QTLs for anther and style morphology. One of them (se2.1) was mapped on chromosome 2 and finely delineated within a short region. Within that region, loci for SL (Style 2.1) and stamen length were confirmed. Style2.1 was cloned and turned out to encode a transcription factor regulating cell elongation; a mutation in the promoter region of Style2.1 was responsible for low activity of the gene during flower development (Chen et al., 2007). Our co-localized QTLs on chromosome 2 for SL and AL under heat were mapped around the same position as se2.1, suggesting that mechanisms responsible for anther and style development under normal and high temperature conditions are at least partially conserved. The two other QTLs related to SP and SL on chromosome 1 and 3 did not co-localised with any previously identified QTLs under normal temperature, indicating additional, distinct mechanisms for style development upon heat stress that lead to SP.

Formation of inflorescences and flowers

The total number of flowers of one plant was reported to correlate with yield per plant under CMH condition (Bhattarai et al., 2016). We examined the flower production by assessing the IN and FPI. The positive correlation between these two traits suggests a common physiological basis and is in line with co-localization of the main QTLs, qIN1 and qFPI1, on chromosome 1. A number of studies were done on reproductive traits under normal conditions and QTLs responsible for flowering time and FPI were published several times (Doganlar et al., 2002; Georgiady et al., 2002; Grandillo and Tanksley, 1996). Among these, Grandillo and Tanksley (1996) reported that one of the QTLs for days to first flower was close to RFLP marker TG125 on chromosome 1. Later, a QTL for FPI was also found to be close to TG125 (Doganlar et al., 2002). This marker is very close to the QTLs identified here, suggesting that inflorescence and flower production under normal and heat condition are controlled by the same genes. Indeed, we previously found that the IN was not significantly influenced by CMH (Chapter 4). The FPI was reduced by heat (Chapter 4), but all genotypes, including the ones used as parents here, reacted similarly (i.e. no genotype-temperature interaction), making it unlikely that heat-specific QTLs could be identified.
References


Supplementary figure and table.

Figure S1. LOD profiles for all investigated traits.
For each trait, LOD value was shown as a curve over the 12 chromosomes of tomato. The horizontal line indicated significance threshold. PV, pollen viability; PN, pollen number; SP, style protrusion; AL, anther length; SL, style length; FPI, flowers per inflorescence; IN, Inflorescence number.
Table S1. Primer sequences for SNP genotyping by KASP assay.

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<th>Chromosome</th>
<th>Primer_AlleleFAM</th>
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(continued)
CHAPTER 6

General discussion
A better understanding of pollen heat-sterility

Crop plants, being sessile organisms, continuously face biotic and abiotic stressors throughout their life cycle, and one of them is heat. Temperatures rising higher than plant’s optimal thresholds cause so-called heat stress, which adversely influences their growth and development (Wahid et al., 2007). Heat exposure at different developmental stages leads to various types of damage. In green leaf tissue, for example, a major effect is the loss of membrane integrity and reduced photosynthesis (Bar-Tsur et al., 1985; Camejo et al., 2005; Kuo et al., 1992). At the reproductive stage, when the effect of high temperature is considered to be most detrimental (Hedhly et al., 2009; Zinn et al., 2010), development of pollen is disturbed, which can be translated directly to lower crop yield (Bac-Molenaar et al., 2015; Dane et al., 1991; Harsant et al., 2013; Madan et al., 2012). Therefore, it is of practical importance to understand “why pollen is sensitive to heat”, and this is the main research question of this thesis.

Inside microsporangium of the anther, pollen formation initiates from the pollen mother cell which undergoes meiosis to give rise to a tetrad of spores. Subsequently, these microspores separate and gradually develop into mature pollen grains (McCormick, 2004). In tomato, we determined that pollen development at 11-9 days before anthesis (DBA) was sensitive to mild heat (MH; Chapter 2) and showed that this time window covered the transition from meiosis to early microspores, refining results from previous studies (Iwahori and Takahashi, 1964; Sato et al., 2002). We also showed that MH lasting for 4 days was sufficient to cause significant loss of pollen viability, while 1 or 2 days MH did not strongly impair pollen development. As pollen viability is essential for successful fertilization, and thereby for crop productivity, its heat susceptibility has received substantial research interests. Results obtained so far enable posing various hypotheses about the factors that may be involved in this phenomenon, such as an inadequate heat stress response (HSR), and/or energy depletion and accumulation of reactive oxygen species (ROS) (Müller and Rieu, 2016).

The HSR, characterised by activation of heat shock protein (HSP; chaperone) genes via heat stress transcription factors (HSFs), and the unfolded protein response (UPR) in the endoplasmic reticulum are thought to maintain protein homeostasis and are essential under high temperature (Fragkostefanakis et al., 2016a; Howell, 2013; Wang et al., 2004). The genes involved were rapidly induced upon heat exposure (Chang et al., 2007; Giorno et al., 2010; Li, 2015), but reportedly less so in developing pollen (Fragkostefanakis et al., 2016b; Gagliardi et al., 1995; Volkov et al., 2005). We found that their transcript level remained normal or even became reduced at the recovery phase after MH (Chapter 2). This may be connected to heat sensitivity, but such an expression pattern does not necessarily mean declined ability to rescue unfolded protein, instead it may well reflect a negative
feedback, by which chaperone protein accumulates during heat represses their expression (Hahn et al., 2011; Morimoto, 1998). Further examination on protein level will be helpful to test this. Protein stability is influenced not only by levels of chaperones, of which there are numerous in plants, but also by the whole biochemical context. Thus, to determine whether the HSR and UPR are less active under high temperature in pollen than in other cell types, rather than looking at expression and levels of HSR and UPR related proteins, in-vivo protein stability assays will be needed for example using fluorescent labelling approaches (Dhar et al., 2011; Hsieh et al., 2014). However, over-activation of the HSR in the tapetum was recently shown to improve thermotolerance of developing pollen to a single hot day (10 h 38°C; Li, 2015), but we found that it did not improve tolerance to MH treatment (data not shown). Therefore, we tentatively propose that protein unfolding is not a major factor in pollen failure upon MH.

The high number of mitochondria in pollen and tapetum (Lee and Warmke, 1979) indicates that these cells are metabolically very active and suggests an important role for energy metabolism to ensure proper pollen formation. The concentration of carbohydrates, the main energy supply, were affected by long-term MH (Pressman et al., 2002; Sato et al., 2006) and this is in line with our observation that fewer and smaller starch granules were present in binucleate pollen after a 4-day MH treatment (Chapter 2). In addition, several days before phenotypic deviations in starch granule accumulation became apparent, contrasting expression of cell wall invertase (CWIN) was found between the damaging 4- and non-damaging 1-day MH. CWIN catalyses the breakdown of sucrose into glucose and fructose, which are then metabolised further or used as energy source by pollen. Since recovery from abiotic stress requires the investment of extra energy (Avin-Wittenberg et al., 2012; Ghosh and Xu, 2014; Jacoby et al., 2011), high expression of CWIN after 1-MH may fulfil this requirement, while normal or reduced CWIN from MH enduring for 4 days and longer (Chapter 2; Sato et al., 2006) may not provide enough sugar to drive the recovery. Therefore, we speculate that abnormal starch deposition is related with insufficient invertase activity. Studying the changes in invertase expression and activity at tissue level, and testing the effect of carbohydrate availability or enhanced invertase activity in developing pollen or tapetum on MH tolerance of developing pollen can provide important answers in this direction.

A high number of mitochondria and strong metabolic activity also suggest pollen and tapetum can be an active site of ROS production. In vegetative tissue, high temperature induces ROS production and thereby oxidative damage to biomacromolecules (Suzuki et al., 2013; Wahid et al., 2007; Wu et al., 2012). High temperature defects in developing pollen and tapetum to some extent resemble the phenomenon of cytoplasmic male sterility (Müller and Rieu, 2016), which was recently linked to mitochondrial function and ROS activity (Hu et al., 2014). So far, however, there has not been strong data regarding ROS
level and localization in heat-stressed pollen or anthers. As alternatives, we measured the oxidative damage to lipids and surprisingly did not find differences upon MH treatment (Chapter 3). MH and heat shock, however, induced the expression of ROS scavengers in anther and developing pollen (Chapter 3; Bita et al., 2011; Frank et al., 2009), as well as antioxidant enzyme activity (Chapter 3). In addition, exogenous application of antioxidants and rescue of protein damage by over-expression of glutaredoxin gave rise to enhanced pollen thermotolerance (Chapter 3; Fahad et al., 2016; Kumar et al., 2014). Therefore, there is some evidence indicating a role of ROS in pollen heat sensitivity. Direct ROS measurements, which may be realized by genetically modified fluorescent sensors (Fujikawa et al., 2016; Meyer and Brach, 2009), will be an important step in solving this issue.

Instead of constituting independent effects, it seems reasonable that the aforementioned factors interact to explain why pollen is vulnerable to heat. Although somewhat speculative, two models may be envisioned. Firstly, high temperature may results in accumulation of ROS in the tapetum and/or developing pollen cells, leading to reduced invertase expression and thus activity. The resulting decrease in available carbohydrates then disturbs the normal process of pollen development, which becomes apparent as abnormal starch accumulation, and more severely, loss of viability. However, insufficient glucose availability may also induce mitochondrial ROS generation (Xiang et al., 2011), thus potentially putting the reduction in invertase activity upstream of ROS accumulation in this model.

As an alternative to uncover the mechanisms behind a certain phenotype, genetic diversity, which enables plant species to exist in diverse habitats, can be used in forward genetic studies. Indeed, genotypes exhibit variable reproductive heat tolerance (Bac-Molenaar et al., 2015; Opeña et al., 1992; Warrag and Hall, 1983). Considerable phenotypic variation upon heat was also observed in a set of tomato cultivars here and relations between heat traits are observed. For instance, pollen viability was positively correlated with fruit set (Chapter 4; Dane et al., 1991). Phenotypic variations in a common environment enable the identification of causal genetic factors, which, in turn, can be useful in uncovering the molecular mechanisms of the traits. A number of quantitative trait loci (QTLs) conferring reproductive heat tolerance have been identified from several species (Bac-Molenaar et al., 2015; Grilli et al., 2007; Lin et al., 2010; Lucas et al., 2013; Opeña et al., 1992), but only in rice, two QTLs accounting for 9-15% phenotypic variation were reported for pollen fertility under heat (Xiao et al., 2011). Here, we found a single, highly significant QTL for pollen fertility in tomato, which explained 36.3% phenotypic variation (Chapter 5). Thus, a single mutation may be responsible for the variation and cloning of the underlying gene will be of significance for understanding pollen heat tolerance. Analysis of near isogenic lines can already give indications as to whether and how the gene fits in the model presented above.
Helping crop plants to cope better with heat

The productivity of several major crops, including wheat, rice and maize, is negatively associated with temperature increase (Challinor et al., 2014; Lobell et al., 2007). To minimize the negative effects, adaptation strategies are implemented in farming practice targeting cultivation management, as well as the choice of most suitable cultivars (Challinor et al., 2014; Driedonks et al., 2016). Thus, the development of crop cultivars that are tolerant to heat environment regarding reproduction is of agronomical importance to ensure food security. The knowledge presented in this work touches upon three areas of practical application, namely molecular breeding, genetic engineering and chemical priming.

Molecular breeding

Introduction of molecular breeding concepts into conventional methods for biotic and abiotic resistance breeding has been proved to be advantageous in terms of throughput and cost (Ashkani et al., 2015; Kumar et al., 2013). Molecular breeding, however, depends on understanding of genetic architecture of a desired trait, namely the causal chromosomal segments/genes, their locations, effects and interactions (Dekkers and Hospital, 2002). Studies in tomato showed that pollen viability was a major determinant for fruit set under heat, but additional factors were female fertility, stigma position, pollen number and anther dehiscence (Akhtar et al., 2012; Dane et al., 1991; Rudich et al., 1977). The results of the QTL analysis in this thesis contained genetic information for several traits (Chapter 5). For the most relevant QTLs, alleles at the same locus worked additively and no epistatic interactions were found, implying that QTL effects are likely to be transmitted to progenies predictably. As further support, the heat tolerance of fruit setting of the cultivar Nagcarlang showed a high combining ability in two independent studies (Bhattarai et al., 2016; Dane et al., 1991). Thus, taking into account what the limiting factors are for a specific genotype, introgression of one or more of the QTLs identified here may substantially improve fruit set under heat.

An important issue in knowledge transfer is how scientific findings are representative for the applied setting. With this in mind, screenings, genetic analyses and QTL mapping for tomato heat tolerance were commonly performed under field or greenhouse condition (e.g. Bhattarai et al., 2016; Dane et al., 1991; Grilli et al., 2007; Kamel et al., 2010; Levy et al., 1978; Lin et al., 2010). However, the unstable environment, such as fluctuation of temperature, may reduce the power of the analysis. To ensure efficient estimation of the genetic effect on the phenotype, in our studies we used growth chambers with a highly stable temperature regime. The obvious drawback then is that the validity of the findings for horticultural settings needs to be confirmed. As a hint that our findings may remain
valid, high correlation was found between fruit setting under field and greenhouse heat conditions among a large set of genotypes and cultivar Nagcarlang was identified as heat-tolerant in this respect in multiple field studies (Dane et al., 1991; Opeña et al., 1992). Altogether, we conclude that QTL analysis has the potential to provide powerful tools for heat-tolerance breeding in tomato.

**Genetic engineering**

As an alternative to breeding, production of abiotic stress resistant plants by genetic engineering relies largely on identification and functional understanding of key players and processes responsible for a certain phenotype. Taking heat tolerance as an example, a number of such players were identified, which belong to the HSF-HSP pathway, redox homeostasis, osmotic adjustment, membrane integrity (Grover et al., 2013; Kotak et al., 2007; Mittler et al., 2012). Concomitantly, transgenic plants with modification of these key components exhibited better heat tolerance (reviewed by Fragkostefanakis et al., 2015; Grover and Singh, 2008; Grover et al., 2013). Particularly for reproductive performance, the over-expression of *HSFA2* and *HSP100* enhanced the pollen tolerance to heat during or at the end of development (Burke and Chen, 2015; Li, 2015). Ectopic expression of *Arabidopsis* glutarerexin (GRXS17) in tomato was reported to enhance vegetative heat tolerance by improvement of ROS scavenging (Wu et al., 2012) and we found that pollen from these transgenic lines was more tolerant than wild type under MH condition (Chapter 3). In addition, based on our results on the comparison between control and MH anther transcriptomes, CWIN, members of UPR pathway, and ascorbate peroxidase 3 will be promising candidates for heat tolerance modulation.

Improvement in functional understanding of key players in heat tolerance is paralleled by progress in genome editing technologies based on sequence-specific nucleases, e.g. zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated proteins (CRISPR/Cas) (Kim and Kim, 2014), which considerably facilitates the generation of new traits (reviewed by Belhaj et al., 2015; Kim et al., 2015; Raitskin and Patron, 2016). An interesting feature of CRISPR/Cas9 system is the possibility to avoid the still controversial use of genetically modified organisms (GMOs). In a non-GMO approach, DNA-free delivery of a pre-assembled complex consisting of Cas9 protein and guide RNAs enabled the generation of intended gene mutations (Woo et al., 2015). If these modern techniques can be used to generate or insert pollen or tapetum specific enhancers in promoters of protective genes, important new genetic variation may become available for heat-tolerance breeding.
Priming for thermotolerance

One of the mechanisms by which plants cope with biotic and abiotic stressors is acquiring tolerance through acclimation (also called hardening or priming; Borges et al., 2014; Bruce et al., 2007). For example, the gradual increase of temperature in the morning can help plants to cope better with high temperature in the middle of the day (Charng et al., 2007; Queitsch et al., 2000). The acquired tolerance can be the result of sustained protection or faster and stronger induction of resistance response, both of which benefit plants when stress reoccurs or gets more intense (Borges et al., 2014). However, acclimation may also be the result of previous encounter with a different natural stressor, in a form of cross-acclimation, or with certain chemical compounds (Hossain et al., 2015; Savvides et al., 2015; Yamauchi et al., 2015). The latter enables enhancement of stress tolerance in production practice through chemical treatment of the crop plants. We showed that exogenous application of melatonin might alleviate tomato pollen susceptibility to heat (Chapter 3), similarly to what was found in rice and wheat with other compounds (Fahad et al., 2016; Kumar et al., 2014). Taken together, priming by chemicals can be useful for improving crop pollen fertility under heat. It will be interesting to extend the study to a larger set of potential priming agents. Linking back to the fundamental side of science, insight in the mechanism of action of beneficial compounds can give new insights in the physiology behind pollen thermotolerance.
References

General discussion
Samenvatting

Gedurende hun levenscyclus ondervinden planten biotische en abiotische stress, hetgeen een negatieve invloed heeft op hun groei en ontwikkeling. Voor de landbouw betekent dit dat gewasopbrengsten vaak ver onder het maximaal haalbare liggen. Eén van de belangrijkste stressfactoren die invloed hebben op de plant is hitte. Als gevolg van de opwarming van de aarde, worden er vaker en extremere perioden van relatief hoge temperatuur (hittegolven) verwacht in de komende decennia. Blootstelling aan hoge temperatuur gedurende meerdere dagen tijdens de bloei belemmert de seksuele voortplanting (en aldus de zaad- en vruchtopbrengst van gewassen) omdat het abnormale ontwikkeling van voortplantingseiwafsel veroorzaakt, met name van de helmknoppen en pollen. De studie beschreven in dit proefschrift concentreerde zich op het mechanisme achter warmte-geëxprimeerde afwijkingen in pollen en genetische variatie in hittetolerantie van pollen, waarbij tomaat is gebruikt als modelsoort.

Hitte is van invloed op planten op alle niveaus, van biomoleculen tot aan organismale fysiologie. Voor wat de schadelijke werking op de ontwikkeling van het pollen betreft, wordt er verondersteld dat een aantal processen worden beïnvloed, waaronder meiose, tapetum ontwikkeling, suiker metabolisme en redox homeostase (hoofdstuk 1). Door slaggevend experimenteel bewijs ontbreekt echter en de aannames zijn meestal gebaseerd op studies van verschillende modelsoorten en temperatuur regimes, waardoor het moeilijk is om algemene conclusies te trekken. Natuurlijke variatie in pollen thermotolerance kan enerzijds nuttig voor plantenveredeling zijn en anderzijds kan het dieper inzicht geven in moleculaire mechanismen. Tot dusver is weinig bekend over genetische determinanten van hittetolerantie van pollen.

In hoofdstuk 2 is het interval tijdens welke pollen ontwikkeling gevoelig is voor milde hitte (MH) gedefinieerd. Er werd aangetoond dat drie tot vier dagen MH gedurende de pollenontwikkelings fase van meiose tot de jonge microspore voldoende is om de levensvatbaarheid van het volwassen pollen sterk te verminderen. Om het onderliggende probleem beter te begrijpen, werd cytologische analyse uitgevoerd op een tijdreeks van helmknoppen volgend op blootstelling aan een controle of een schadelijke 4 dagen MH behandeling in de gevoelige fase. Terwijl in de eerste dagen na de behandelingen geen verschillen in helmknop- en pollenontwikkeling werden waargenomen, was ongeveer een week later, dat wil zeggen net na de pollen mitose 1, 50% van de pollen in de meeldraden van de MH behandeling dood en werden er bij de nog levende pollenkorrels afwijkingen geconstateerd in de zetmeelkorrels en grootte van het pollen. Microarray analyse werd toegepast op bloemen die werden blootgesteld aan een controle behandeling, een niet-schadelijke 1-daagse MH of een schadelijke 4-daagse MH behandeling. Hieruit werd geconcludeerd dat de celwand invertases, die betrokken zijn bij de opname van suikers.
in metabolisch actieve weefsels, een aandeel kunnen hebben aan pollenabortus door hitte.

Er is aangetoond dat de schade als gevolg van hitte in het vegetatieve weefsel wordt veroorzaakt door toename van de concentratie zuurstofradicalen (ROS). **Hoofdstuk 3** beschrijft een poging om te testen of dit ook voor pollen geldt. Alhoewel er geen verhoging werd gevonden in peroxidatie van membraanlipiden, een vorm van oxidatieve schade, werd er tijdens en na de MH behandeling een verhoging gevonden in de expressie en activiteit van verschillende enzymen die zuurstofradicalen onschadelijk maken. Gegeven dat de activiteit van dergelijke enzymen vaak wordt gestimuleerd door accumulatie van zuurstofradicalen, kan dit wijzen op verstoring van redox homeostase door hitte. Toediening van melatonine (dat hydroxyl en $\text{H}_2\text{O}_2$ radicalen opruimt) en verhoogde expressie van glutaredoxin (dat geoxideerde eiwitten reduceert) verbeterde de pollen kwaliteit na MH behandeling. Er zijn dus aanwijzingen dat zuurstofradicalen een rol spelen bij hitte-geïnduceerde pollenafwijkingen, maar sterke conclusies kunnen nog niet worden getrokken.

Correlaties tussen verschillende eigenschappen in een verzameling van genotypen kunnen duiden op een hiërarchische afhankelijkheid of op onderliggende gemeenschappelijke mechanismen. In **hoofdstuk 4** werden reproductieve eigenschappen van een aantal tomaatrassen geanalyseerd onder normale en onafgebroken MH condities. Hitte had grote gevolgen voor de meeste van de bestudeerde eigenschappen en er waren aanzienlijke verschillen tussen de rassen voor wat betreft het effect van hitte. Analyse van relaties tussen eigenschappen duidde erop dat levensvatbaarheid van pollen, maar niet de vrouwelijke vruchtbaarheid, een beperkende factor is voor het produceren van vruchten met zaden onder MH. Rassen met tegengestelde uitkomsten onder MH werden geïdentificeerd voor verdere genetische studie.

Ondanks het belang van levensvatbaarheid van pollen voor reproductie onder hoge temperatuur, zijn genetische determinanten voor deze eigenschap alleen gemeld in een studie op rijst. Om de genetische basis van verschillen in hitte tolerantie tussen twee contrasterende tomaatrassen aan het licht te brengen, werd van individuen van een segregerende $F_2$ populatie een aantal eigenschappen gemeten en het genotype bepaald (**hoofdstuk 5**). Correlatieanalyses identificeerden regio’s van het genoom die voor verschillende reproductieve hittetolerantie-eigenschappen verantwoordelijk waren, met inbegrip van de levensvatbaarheid van pollen en het aantal pollen. Het locus dat de levensvatbaarheid van pollen beïnvloedde, werd gelokaliseerd op chromosoom 11; het was verantwoordelijk voor 36% van waargenomen fenotypische variatie tussen individuen. Het feit dat verschillende eigenschappen door dezelfde genoordegeno’s beïnvloed werden, wijst op afhankelijkheid van kenmerken en pleiotropische effecten.
Samengevat, beschrijft deze studie de korte tijdsspanne waarin MH pollenontwikkeling beïnvloedt en laat zien dat de daaruit volgende defecten in pollen pas tot uiting komen in een specifiek, laat stadium van ontwikkeling. Er is aanvullend bewijs gevonden voor de betrokkenheid van suikermetabolisme en accumulatie van zuurstofradicalen, twee factoren die misschien wel van elkaar afhankelijk zijn (hoofdstuk 6). Omdat de helmknop uit verschillende weefsels bestaat, die verschillend kunnen reageren op hitte en zo beperkingen opleggen aan levensvatbaarheid van pollen, zouden toekomstige studies zich moeten richten op weefselspecifieke benaderingen. De duidelijke genetische determinant van pollen-hitletolerantie opent mogelijkheden voor toepassing door moleculaire veredeling. Andere praktische oplossingen kunnen worden gebaseerd op genetische manipulatie van verbeterde redox homeostase of chemische voorbehandeling.
Summary

During their life cycle, plants encounter a suit of biotic and abiotic stresses, which negatively affect their growth and development. In an agricultural setting, this means that crop yields are commonly well below what is maximally attainable. One of the major stress factors impacting plant performance is heat. As a result of global warming, periods of relatively high temperature (i.e. heat waves) are predicted to occur more frequently and become more severe in the coming decades. Exposure to a multiple days of high temperature during flowering hampers plant reproduction (and thus yield of seed and fruit crops) due to abnormal development of reproductive tissues, especially anthers and the pollen contained within them. The study presented in this thesis focused on the mechanism behind heat-induced pollen failure and genetic variation for pollen thermotolerance, using tomato as a model species.

Heat affects plants at all levels, from biomolecules to organismal physiology. Regarding the deleterious effect on pollen development, a number of processes have been proposed to be affected, including meiosis, tapetum development, sugar metabolism and reactive oxygen species (ROS) homeostasis (chapter 1). However, strong experimental evidence is lacking and the suggestions are mostly based on studies using different species and temperature regimes, making it hard to draw general conclusions. Natural variation in pollen thermotolerance may on one hand be useful for plant breeding purposes and on the other hand give further insight in molecular mechanisms. So far, little is known about genetic determinants of pollen thermotolerance.

In chapter 2, the window during which pollen development is sensitive to mild heat (MH) was defined. Three to four days of MH around the meiosis to early microspore phase of pollen development was shown to be sufficient to cause severe reductions in viability of the resulting mature pollen. To better understand the underlying problem, cytological analysis was performed on a time series of anthers from the days following exposure to a control or a damaging 4-day MH treatment at the sensitive stage. While in the first days after the treatments no differences in anther and pollen development were observed, about one week later, i.e. just after pollen mitosis I, 50% of pollen in the MH-treated anthers was dead, and abnormalities in starch granules and pollen size were found in the remaining living pollen grains. Microarray analysis was applied to flowers that received either control, non-damaging 1-day MH and 4-day MH treatments. It was concluded that cell wall invertases, which are involved in unloading of sugars into sink tissue, may play an role in heat caused pollen abortion.

ROS have been demonstrated to mediate heat induced damage in vegetative tissue. Chapter 3 describes an effort to test whether this also applies to pollen. Although no increase was found in membrane lipid peroxidation, a type of ROS damage, expression
and activity of several ROS scavengers were found to be induced during and after MH treatment. Because ROS scavenger gene activity is often stimulated by ROS accumulation, this might indicate disturbance of ROS homeostasis upon heat. Furthermore, application of melatonin (which scavenges hydroxyl and H$_2$O$_2$ ROS), and enhanced expression of a glutaredoxin (which reduces oxidised proteins) improved pollen performance after MH treatment. Thus, there are indications for a role of ROS in heat-induced pollen failure, but strong conclusions cannot be drawn as yet.

Correlations between performances of different traits in a set of genotypes may suggest a hierarchical dependency or point at common underlying mechanisms. In chapter 4, a set of tomato cultivars was analysed for performance of reproductive traits under control and continuous MH condition. Heat had significant effects on most traits under study and considerable variation in performance and treatment effects was observed among cultivars. Trait correlation analysis suggested that pollen viability, but not female fertility, is a limiting factor for seeded fruit set under MH condition. Cultivars with contrasting performances under MH were identified for further genetic study.

Despite the importance of pollen viability for reproduction under high temperature, genetic determinants for this trait have only been reported in one study on rice. To reveal the genetic basis of differences in thermotolerance between two contrasting tomato cultivars, individuals from a segregating F$_2$ population were phenotyped and genotyped (chapter 5). Correlation analyses identified QTLs for different reproductive thermotolerance traits, including pollen viability, pollen number. The locus affecting pollen viability was located on chromosome 11 and accounted for 36% of observed phenotypic variation among individuals. The co-localization of QTLs for several traits pointed at trait dependencies and pleiotropic effects.

Taken together, this study narrowed down the time frame in which MH affects pollen development and revealed that pollen defects become apparent at a specific, late stage of development only. Further evidence was found for involvement of sugar metabolism and ROS activity, two factors that might well depend on each other (chapter 6). Because the anther consists of different tissues, which may differentially react to heat to impose constraints on pollen viability, future studies should apply tissue-specific approaches. The clear genetic determinant of pollen thermotolerance opens possibilities for application through molecular breeding. Other practical solutions could be based on genetic engineering for improved ROS homeostasis or chemical priming.
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Curriculum Vitae

Jiemeng Xu was born on October 22, 1987 in Zhangjiajie, Hunan, China. After the primary and secondary education from hometown, he enrolled as a bachelor student in Hainan University at 2005. During the bachelor, he majored in agriculture and studied crop physiology, pathology, cultivation, breeding, etc. At 2009, he obtained the bachelor degree and started master study which focused on mapping the genes responsible for thermos-sensitive genic male sterility in rice and genetic engineering of tobacco with suppressed later buds growth. Afterwards, with the fellowship from China Scholarship Council, he moved to the department of Molecular Plant Physiology in Radboud University, Nijmegen for Ph.D. study under the supervision of dr. Ivo Rieu and Prof. dr. Titti Mariani. His doctoral project concentrated on the underlying molecular mechanisms of heat-induced pollen failure and genetic variation of pollen heat-tolerance in tomato.
Publications


