Propositions

1. Since tomato pollen is sensitive to temperature variations, results from climate chamber experiments with controlled temperature cannot be extrapolated to pollen thermo-tolerance in commercial tomato production.
   (this thesis)

2. The pollen isolation protocol is a critical determinant for the success of pollen metabolomics studies.
   (this thesis)

3. The choice of the p-value has an undesirably strong influence on a study's conclusions.

4. Nutrition should be considered as the first medicine.

5. Meatless Monday is an effective policy to reduce global warming.

6. The health of science rests on scientist's honesty.

7. If Dutch were not Dutch but French, most of them would not speak English

Propositions belonging to the thesis, entitled

"Identification of metabolites involved in heat stress

response in different tomato genotypes"

Marine Joséphine Paupière

Wageningen, 12 June 2017

Identification of **metabolites** involved in **heat stress** response in different **tomato** genotypes

Marine J. Paupière

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Identification of metabolites involved in heat stress response in different tomato genotypes

Marine J. Paupière

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Contents

Chapter 1

General introduction

Climate change

Climate is unequivocally changing. In 2014, IPCC released the fifth assessment report on climate change and stated that "the atmosphere and ocean have warmed, the amounts of snow and ice have diminished and sea level has risen" (IPCC 2014). The main cause for this global warming is extremely likely to be due to anthropogenic activities that are widely acknowledged by climate specialists (Cook et al. 2013). Heat waves and extreme temperatures are expected to be more frequent and to last longer (Meehl and Tebaldi 2004). This climate change is expected to affect the world of today in many ways, including the extinction of species that cannot escape their environment and a decrease in food productivity. For instance, the well documented heat wave of 2003 in Europe led to a yield loss of 25% and 21% for maize and wheat in France, respectively, with an overall economic loss in the European Union estimated at 13 billion euros (IPCC 2007). The direct effect on the human population is not to be underestimated, especially if one takes into account that the world population is predicted to rise from 7.3 billion up to 11 billion by the middle of the 21st century, thus alarming the decision-makers (Porter and Semenov 2005). From the reduction of greenhouse gas emission with the Kyoto protocol (Oberthur and Hermann 1999) to the recent COP21 agreement to limit the temperature increase of 2˚C (Iyer et al. 2015), policymakers mull over mitigation options. A synergy of policies and adaptation strategies may enhance the efficacy to respond to climate change and limit the consequences on living organisms and ecosystems. The adaption of crops is nested in the identification of genotypes tolerant to temperature increases and in a greater understanding of high temperature plant responses that will sustain crop production under climate change.

Plant responses to high temperatures

The physiological response to heat stress depends on how an individual experiences the rise of temperatures. High temperatures in Central Europe might not be qualified as "high" in another part of the globe. The response to heat stress relies on the individual level of tolerance to withstand high temperatures. Heat stress tolerance is determined by the threshold temperature above which deleterious effects in an individual will appear (Wahid et al. 2007). For instance, the threshold temperature of the crop cotton is 45°C, which is 15°C higher than the one of the crop tomato, before the appearance of negative effects (Wahid et al. 2007). Lichtenthaler 1996 introduced the stress concept in plants and reviewed the different definition given to stress. For instance, Levitt 1980 determined stress as "any environmental factor potentially unfavourable to living organisms" and Larcher 1987 defined stress in a way that "every organism experiences stress, although the way in which it is expressed differs according to its level of organization". In line with this, the stress response aimed to adapt to the adverse condition and to reach a new level of homeostasis, in order to survive the stress condition (Figure 1). Tolerant genotypes are those that succeed to adapt and survive, whereas sensitive genotypes fail to do so and experience deleterious negative effects of the stress. In this thesis, heat stress and high temperatures are considered as temperatures above the optimal growing temperatures that lead to biological responses. Heat stress consequences do not only depend on the extent of the temperature increase, but also on the duration of the stress (or degree days) (Mesihovic et al. 2016). A short heat stress of 32°C for a few hours is not experienced in the same way as a long heat stress of the same temperature for several days. Furthermore, consequences of a heat stress may differ

depending on the developmental stage of a plant at the moment when the stress takes place. For example, plant reproduction has been demonstrated to be extremely sensitive to high temperatures compared to the vegetative growth phase (Zinn et al. 2010). Different plant organs and tissues may reveal different reactions in response to a heat stress. All these factors make the heat stress response a complex system to investigate. In our research, we used multiple stress treatments, ranging from a short acute heat stress to long moderate heat stress on two reproductive organs: anther and pollen.

When a crop suffers from heat stress, a number of physical symptoms can easily be seen by eye, such as leaves rolling, senescence, discoloration, low seed germination, slower plant growth and a decrease of yield (Wahid et al. 2007). These physical symptoms are the result of a complex mechanism called heat stress response (HSR) that takes place once the rise in temperature has been detected by the plant (Mittler et al. 2012). Mittler et al. 2012 reviewed how plants react to high temperatures by first adjusting the fluidity of their cell membranes (1). The change of membrane fluidity leads to activation of calcium channels (2). The influx of calcium into the cell induces a strong transduction signal that leads to the production of reactive oxygen species (ROS) and the activation of transcription factors (3). Among these transcription factors, the well-known heat shock factors (HSFs) are found (Scharf et al. 2012). They induce the production of heat shock proteins (HSPs), one of the key players of the HSR mechanism. HSPs are able to re-fold denaturated proteins that appear under high temperatures and also to prevent protein aggregation (Vierling 1991). In addition to the rapid accumulation of HSPs and ROS, specific metabolites are known to accumulate under heat stress, such as antioxidants and osmolytes (Wahid et al. 2007) (4). ROS are important components of the stress signalling cascade, but they are also harmful for the cell, since they are very reactive and can induce lipid peroxidation and membrane oxidation (Driedonks et al. 2015). Hence, the production of antioxidants that have ROS scavenging properties are needed to maintain ROS homeostasis. Whether or not the response to the heat stress is sufficient for the plant to survive the unfavourable conditions depends of its tolerance threshold.

Tolerance definition

Not all plants have the ability to survive heat stress. A large variation of tolerance is found between species, within the accessions of a species and also between the organs and tissues of a plant. The tolerance to high temperatures is divided in two categories (i) basal tolerance and (ii) acquired tolerance (Bokszczanin et al. 2013). The basal tolerance is described as the ability to survive high temperatures without previous acclimation; it is also considered as an evolutionary adaptation that can be observed in some species as specific phenotypic characteristics, such as the thickness of the leaves which controls transpiration, changing leaf orientation and a longer root system (Bita and Gerats 2013). The acquired tolerance differs from the basal tolerance in the sense that an increased tolerance is obtained through acclimation, which is induced by a non-lethal stress preceding a lethal stress, or by a gradual increase of temperatures. Surviving heat stress has been linked to the ability to maintain a high photosynthesis activity and to accumulate specific metabolites such as proline and glycine betaine that are known to regulate the osmolarity within the cell, and also antioxidants as previously mentioned (Wahid et al. 2007). A proper adaptation and acclimation of the plant's

homeostasis under high temperatures is the key to maintain plant vigour and the production of seeded fruits. The tolerance of a plant is often considered relative to another plant. For example, under a constant day temperature of 32°C the tomato genotype Hazera 3042 produced on average 5.9 fruits per plant with 53% of seeded fruits (Firon et al. 2006). This is therefore considered to be a more tolerant genotype than the genotype Hazera 3017 that produced on average only 2.6 fruits per plant that did not contain any seeds. Nevertheless, this does not mean that the heat stress did not lead to a reduction of fruit production in Hazera 3042 compared to the optimal growing temperatures. Hence, the absolute qualification of tolerance would be the ability of a plant to set fruit under high temperatures as good as it would do at optimal growing temperatures.

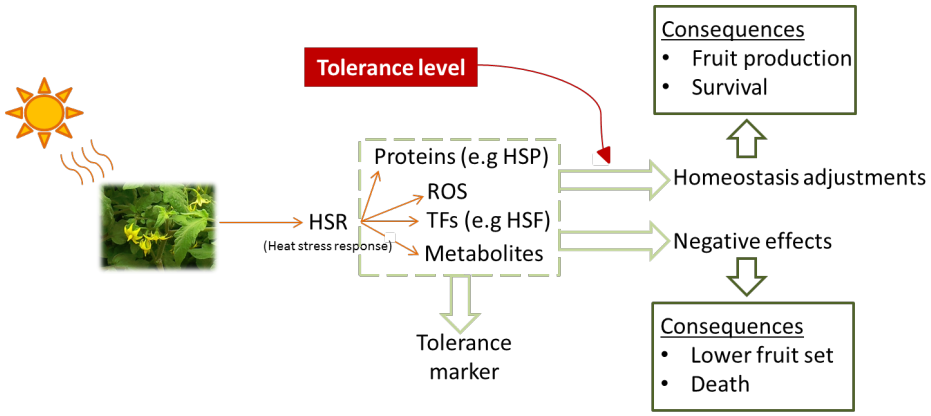

Figure 1. Heat stress response and tolerance. When a plant is exposed to high temperatures the heat stress response (HSR) is activated and leads to an alteration of plant organization at different levels including proteins, transcripts and metabolites. Based on the tolerance threshold of the plant, the plant can either (i) adjust its homeostasis to overcome the environmental stresses by accumulating HSPs, and protective metabolites (e.g. antioxidants) in order to survive or (ii) fail adjusting its homeostasis that might result in accumulation of aggregating proteins, ROS and higher energy demand that leads to permanent negative effects including death. TFs, Transcription Factors; HSP, Heat Shock Proteins; HSF, Heat Shock Factors; ROS, reactive oxygen species

Tomato

Tomato belongs to the Solanaceae family that encompasses several other famous crops, such as potato, eggplant and pepper. Tomatoes are native to South America and were introduced in Europe in the 16th century by the Spanish conquistador Cortés on his way back from the capture of the Aztec city, Tenochtitlan, known as Mexico City (Bergougnoux 2014). During a long time, tomatoes were used as ornamentals and thought to be toxic, but in the 17th century they started to appear in the European cuisine. Today, tomatoes are eaten in diverse manners from raw, cooked, dry to highly processed paste and can easily be found in kitchen gardens. The domesticated tomatoes found on our plate have been extensively bred for diverse traits such as yield, shape, shelf life, pest resistance and more recently taste and nutritional values (Bergougnoux 2014). Tomatoes offer a rich source of vitamin A (lycopene) and C (ascorbate), both antioxidants that have been shown to correlate with prevention of cancer and cardiovascular diseases (Rao and Agarwal 2000). On the recent FAO data of 2013

(http://www.fao.org), tomato is the second most consumed vegetable in the world after potato. The biggest tomato producers are found in Asia, which represents 60.3% of tomato production (Figure 2), but the two countries with the highest yield per hectare are found in Europe, namely Belgium and the Netherlands. In total, Europe represents 12.7% of the world tomato production, with the top producers Spain and Italy. Besides to have invaded the crop market and our plates, tomato is considered by researchers as a model crop due to its attractive traits (Foolad 2007): (i) a relative short life cycle, (ii) a high self-fertility that allows genetic stability over generations, (iii) a sequenced genome and (iv) a large diversity present among wild accessions that, all together, makes tomato an attractive and powerful resource to study tolerance to abiotic and biotic stresses.

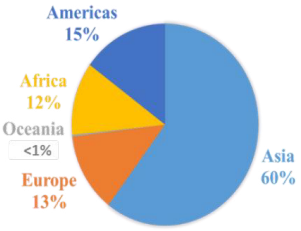

Figure 2. World tomato production in 2013 - FAO

Tomato and high temperatures

The optimal growing temperature of tomato is between 19°C and 25°C (Hurd and Cooper 1970). A temperature of a few degrees above this threshold can lead to serious deleterious effects, such as flower abscission, decrease of pollen quality, abnormal growth, reduced fruit set (Figure 3; Hasanuzzaman et al. 2013). In addition, this temperature stress has a direct impact on the quality of the fruit, such as cracks, blossom end rot, immature fruit or watery tissue (Abdulbaki 1991). For instance, tomato plants exposed to a long heat stress with an average temperature of 34°C/19°C exhibit a flower drop of 34% and a decrease of fruit set up to 71% (Hazra et al. 2009). The production of tomatoes requires fertilization of the female egg by the pollen grain. The development of the pollen occurs inside the flowers, within the anthers (Honys et al. 2006). Anthers are considered as the supportive tissue that supplies the pollen with essential metabolites required for its development (Pacini 1996). Regarding the performance of tomato under heat stress, the pollen grain was demonstrated to be the weakest point, due to its vulnerability to high temperatures which results in a decrease of tomato fruit yield (Bokszczanin et al. 2013). The sensitivity of pollen to high temperatures is associated with a disruption of developmental processes that can occur at different stages was reviewed by Muller and Rieu 2016: (i) at meiosis stage with the alteration of cell division, (ii) at microspore stage with the degeneration of anther tapetal cells which provide pollen nutrition and (iii) during the microspore maturation stage with a failure to accumulate carbohydrates. Genotypes which are tolerant to high temperatures have the ability to maintain a high pollen quality, which results in a greater fruit production compared to sensitive genotypes (Dane et al. 1991). The ability to maintain high pollen quality under heat stress has been mainly attributed to the high abundance of sugars (Firon et al. 2006).

Considering the forecast of an increased incidence of heat waves in Europe, local European producers may face critical situations with a decrease of yield (Meehl and Tebaldi 2004). For instance, reports have mentioned that in Spain the heat wave of 2003 led to a reduction of tomato yield (Pazos 2004). The FAO data between 2002 and 2003 showed a yield drop for tomato of 7% (http://www.fao.org). Hence, the increased temperatures forecasted for the near future (e.g. increase of heat waves), the importance of the tomato crop worldwide, the variation in heat stress response of different tomato accessions and the applicability of tomato as a model plant, makes it a valuable object for the study of the mechanisms of heat tolerance of the whole plant and, in particular, of the male reproductive organs, whose high sensitivity is the bottleneck of fruit production under high temperatures.

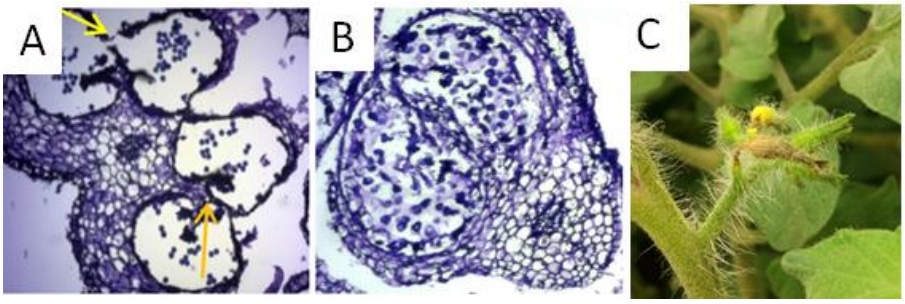

Figure 3. Impact of temperature on tomato flower buds. Pictures A and B are anther cross sections of the tomato model cultivar MicroTom exposed to control (A) and mild long heat stress (B). Under control temperatures (A) four locules are present and at maturity the stonium breaks to allow the release of pollen (yellow arrow) while under heat stress (B) the number of locules are altered and deformed. Cross sections were stained with toluidine blue. Picture C is tomato flower abscission observed under mild high temperatures

The SPOT-ITN consortium

As mentioned above, fertile pollen production has been demonstrated to be the critical point of fruit production. Understanding the male gametophyte's tolerance mechanisms to high temperatures is necessary to ensure sustainable tomato reproduction and, therefore, tomato fruit yield. Such a problem needs to be addressed at a multidisciplinary level, in order to cope with the complexity of the heat stress response, which includes the reprogramming of gene expression, induction of specific proteins such as HSPs, and the metabolic homeostasis maintenance. In 2012, the Solanaceae Pollen Thermo-tolerance – Initial Training Network (SPOT-ITN) consortium (http://spot-itn.eu/) was set up with different research groups from Europe and Israel to address this issue at different levels using a multi-omics approach involving; epigenomics, transcriptomics, proteomics and metabolomics tools. The role of Plant Breeding, Wageningen University & Research in this project was to gain knowledge on the metabolic response of pollen and anthers under changing environments, in order to identify metabolites associated with the tolerance of tomato to high temperatures.

Metabolomics

Metabolites are important chemical components of the plant system. They are involved in many processes, such as functioning as building blocks for more complex molecules, energy generation, storage and distribution, signalling and regulation of development (Arbona et al.

2013). For example, the amino acids alone show a large diversity of use: they can be incorporated into proteins (Pratelli and Pilot 2014), serve as precursor of metabolic pathways (e.g. phenylalanine for phenylpropanoids (Fraser and Chapple 2011)), be part of signalling (e.g. GABA (Ramesh et al. 2016)) or be involved in stress response (e.g. proline as compatible solute (Hayat et al. 2012)). In the plant kingdom 200,000 metabolites are estimated to exist (Fiehn 2002). The expansion of metabolite analysis with the development of untargeted metabolic profiling analysis – metabolomics, allowed a fast exploration of the metabolome in any given tissue. For instance, analysis of a polar extract using a gas chromatography mass spectrometry analysis platform (GC-MS) allows one to obtain an accurate profile of primary metabolome in less than 40 min. Metabolomics platforms offer a valuable tool to explore the plant metabolome and to gain knowledge on the metabolic response to heat stress. In bentgrass (*Agrostis scabra*) leaves, for example, the use of metabolomics has been shown to lead to the identification of metabolites associated with tolerance to high temperatures (Xu et al. 2013). The tolerant accession showed a higher accumulation of hexose sugars, used as energy source, of branched amino acids involved in alternative energy source, of the polyamine putrescine as antioxidant and of diverse compatible solutes such as proline and sucrose to cope with osmolarity regulation (Xu et al. 2013).

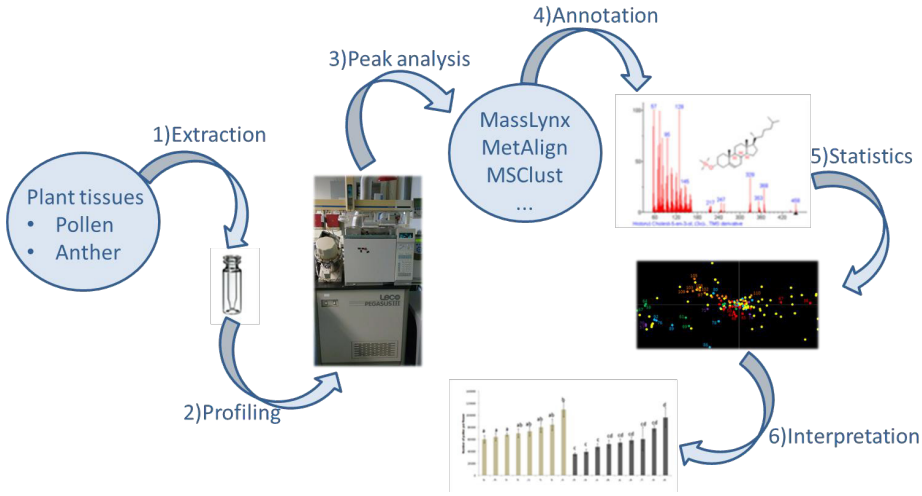

Figure 1. Metabolomics workflow. Metabolomics analysis include (1) the sample preparation, sample analysis with analytical platforms (2), data processing with peak picking, alignment and annotation (3,4), statistical analysis (5) and the data interpretation.

The use of metabolomics approaches applied to male reproductive tissues, especially pollen, in response to heat stress is not yet an established approach. However, particular metabolites have been shown to play a major role in the stress response, as reviewed in Chapter 2 of this thesis. Hence, in our research we used metabolomics approaches to obtain a wider picture of the metabolome of different reproductive organs, including pollen and anthers in different tomato genotypes exposed to heat stress (Figure 4). The reproductive tissues, in particular pollen, possess certain morphological and physiological characteristics that are different from vegetative tissues, for which many metabolomics methods have been developed, such as the

water content, tissue amount, size, diverse physiological states and different morphology during development. We first assessed the suitability of several pollen isolation methods for our metabolomics studies: we addressed the impact of anther contamination and pollen rehydration, which both could occur during pollen isolation and might affect the reliability of the obtained metabolic profiles. Although one metabolomic platform allows the detection of a large panel of metabolites, it is clear that combinations of different platforms will provide a more comprehensive view of the metabolome. In our approach, we mainly used two metabolomics platforms: gas chromatography coupled to mass spectrometry (GC-MS) to study polar primary metabolites (amino acids, sugars and organic acids) and liquid chromatography coupled to mass spectrometry (LC-MS) to detect semi-polar secondary metabolites (phenylpropanoids, polyamines and alkaloids).

Thesis outline

In this project, we used a metabolomics-based exploratory approach to study the metabolic response of different tomato genotypes and their reproductive tissues to exposure of short and long heat stress conditions. This allowed us to identify stress-responsive metabolites and metabolites associated with tolerance to high temperatures. Such metabolites can be used as metabolic markers in breeding.

In the second chapter, we reviewed the current state of the art of the research on pollen metabolites involved in high temperature responses. We offered a breeding strategy to use metabolic markers in the process of developing new tomato varieties tolerant to high temperatures.

In chapter three, we assessed the suitability of available pollen isolation protocols for untargeted metabolic profiling. We focused on drawbacks of the pollen isolation protocol such as (i) pollen rehydration, (ii) enzymatic activities and (iii) anther contamination.

In chapter four, we studied the impact of short acute heat stress on secondary metabolites of developing pollen of the tomato genotype MicroTom. This study revealed that (i) one hour heat stress at 38°C was not sufficient to induce strong metabolic alterations in developing pollen and that (ii) the developmental stages younger than polarized pollen seemed not to have a significant metabolic content compared to later stages, at which the metabolic responses to heat stress are, therefore, likely to occur. Nevertheless, in nature or in growing practice the heat stress condition may take place at any stage of reproductive organ development and may affect the resulting pollen quality. Hence, in order to be able to identify metabolites associated with pollen thermo-tolerance, we conducted our studies under a milder but longer heat stress regime, which allowed us to obtain clearer metabolic effects, without forcing complete tissue deterioration. Since the pollen did not give a strong metabolic response under short heat stress while we have observed such a response in anther, we decided to focus on anther tissue to assess further metabolic changes in response to heat stress.

In chapter five, we phenotyped the pollen quality of 13 tomato accessions under long mild heat stress by recording the amount of pollen produced and pollen viability, in order to identify genotypes tolerant and sensitive to high temperatures.

In chapter six, two genotypes of the 13 described in chapter five with contrasting tolerance background were selected to (i) determine the critical developmental stage that led to a decrease of pollen viability under long mild heat stress and (ii) to explore the metabolome of developing anthers under high temperatures and control temperatures. This approach allowed us to identify metabolites associated with the tolerance to high temperatures.

In chapter seven, I discuss the integration of the different chapters of this thesis in view of sustainable tomato production under changing climate conditions. I also give recommendations on working with pollen in metabolomics and other –omics field. The thesis concludes with a summary of the work and key messages.

Chapter 2

The metabolic basis of pollen thermo-tolerance: perspectives for breeding

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Abstract: Crop production is highly sensitive to elevated temperatures. A rise of a few degrees above the optimum growing temperature can lead to a dramatic yield loss. A predicted increase of 1–3 degrees in the twenty first century urges breeders to develop thermo-tolerant crops which are tolerant to high temperatures. Breeding for thermo-tolerance is a challenge due to the low heritability of this trait. A better understanding of heat stress tolerance and the development of reliable methods to phenotype thermo-tolerance are key factors for a successful breeding approach. Plant reproduction is the most temperature-sensitive process in the plant life cycle. More precisely, pollen quality is strongly affected by heat stress conditions. High temperature leads to a decrease of pollen viability which is directly correlated with a loss of fruit production. The reduction in pollen viability is associated with changes in the level and composition of several (groups of) metabolites, which play an important role in pollen development, for example by contributing to pollen nutrition or by providing protection to environmental stresses. This review aims to underline the importance of maintaining metabolite homeostasis during pollen development, in order to produce mature and fertile pollen under high temperature. The review will give an overview of the current state of the art on the role of various pollen metabolites in pollen homeostasis and thermo-tolerance. Their possible use as metabolic markers to assist breeding programs for plant thermo-tolerance will be discussed.

Keywords: pollen; heat stress; thermo-tolerance; high temperature; metabolite; breeding

1. Introduction

Environmental stresses are important factors affecting worldwide crop production (Mittler 2006). Among them, high temperature plays a crucial role and this review will focus on this specific abiotic stress (Ainsworth and Ort 2010). Heat stress is a complex trait which depends on the duration, the fluctuation and the intensity of temperature rise above the optimal growth temperature (Wahid et al. 2007). High temperature is one of the major factors limiting the growth season in many parts of Asia, one of the most important producers of daily consumed crops such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), potatoes (*Solanum tuberosum*) and tomatoes (*Solanum lycopersicum*). The expected global warming, with a predicted increase of 1–3 °C during the twenty first century, could therefore have a major impact on agriculture and may lead to significant decreases in crop production (IPCC 2012). Breeding for thermo-tolerant genotypes is of major importance to maintain crop production under hot conditions. This requires a thorough understanding of the mechanisms underlying heat stress tolerance.

Plants can respond to heat stress through different mechanisms. High temperature alters plant growth, including rolling of leaves, leaf senescence, root and shoot growth inhibition as well as seed germination reduction, fruit discoloration, decrease in pollen viability and decrease in yield (Hasanuzzaman et al. 2013), as a result of severe alterations in basic physiological processes, such as increased respiration, decreased photosynthesis, increased membrane permeability and ROS production (Figure 1). A genotype is considered thermo-tolerant when it can produce economic yield under heat stress. Several experimental parameters can be measured to monitor thermo-tolerance (Wahid et al. 2007), including cell membrane thermostability, photosynthesis activity, pollen viability and fruit set. These parameters will be discussed below.

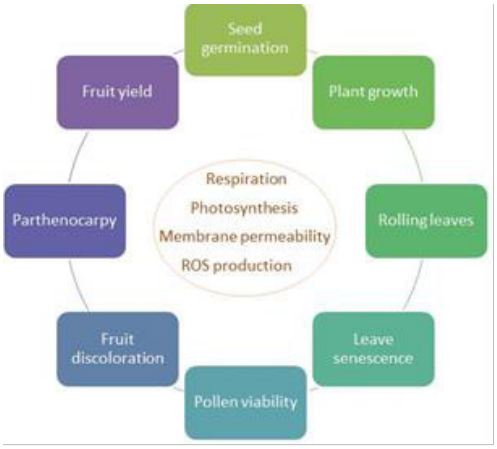

Figure 1. General effects of heat stress on plant physiology.

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(i) The alteration of membrane structures under high temperature is a common response to heat stress. High temperatures alter membrane permeability by increasing membrane fluidity.

This leads to an increase of electrolyte loss which is an indication of decreased cell membrane stability (Wahid et al. 2007).

(ii) Photosynthesis is highly sensitive to high temperature (Allakhverdiev et al. 2008). The alteration of the thylakoid membrane under heat stress is directly linked to a decrease of photosystem II activity which affects the rate of photosynthesis (Yamamoto et al. 2008). In addition, the sensitivity of the enzyme Rubisco to high temperature can lead to a decrease in carboxylase activity which will also inhibit photosynthesis (Demirevska-Kepova and Feller 2004) and likewise CO2 fixation. Consequently, the ability to assimilate CO2 under heat stress conditions is associated with thermo-tolerance.

(iii) Reproduction has been demonstrated as the most heat sensitive process in plants as reviewed in two recent papers(Bokszczanin et al. 2013, De Storme and Geelen 2014) (Figure 2). Many studies have shown that pollen quality is the most important determinant of fruit production under heat stress (Dane et al. 1991, Firon et al. 2006). Therefore, analysis of pollen viability and fruit set under high temperature is a direct trait for the study of thermo-tolerance in plants.

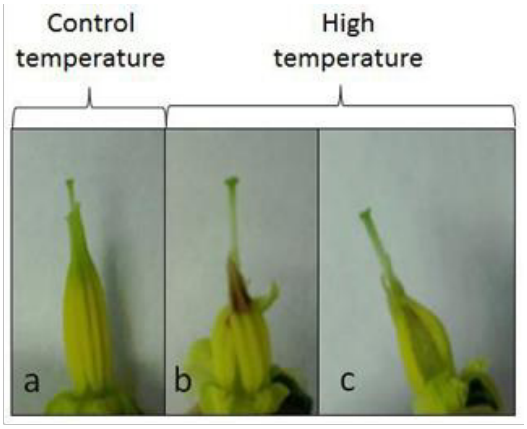

Figure 2. Effect of high temperature (34 °C/28 °C) on flowers of *Solanum lycopersicum* cv. Nagcarlang. Pictures represent mature flowers under control conditions (**a**) and high temperature (**b**–**c**). Under high temperature, anthers showed deformation, dark coloration of the anther tip and elongated pistils. Those flowers had a low percentage of pollen viability (<10%).

Heat stress induces a reorganisation of the transcriptome (Frank et al. 2009), proteome (Li et al. 2013) and metabolome (Kaplan et al. 2004) which can either lead to failures in plant development, or instead to acclimation to high temperature (Bokszczanin et al. 2013). On the one hand, metabolic alterations caused by heat stress can lead to severe damage in sensitive plants compared to tolerant plants. For example, high temperature can lead to a decrease of antioxidant enzyme activity (Djanaguiraman et al. 2010). This will decrease the plant's ability to protect itself against reactive oxygen species and leads to lipid peroxidation of cellular membranes. In addition, the alteration of photosynthesis activity under high temperature can lead to a decrease of sugar abundance (Chaitanya et al. 2001). Sugars are primary metabolites essential as precursors for different metabolic pathways and plant nutrition. On the other hand, plants have the ability to respond to heat stress by inducing or activating protective mechanisms.

For example, most of the transcripts induced in response to high temperature encode heat shock proteins (HSPs) (Rizhsky et al. 2004).

HSPs are proteins which are rapidly produced under heat stress and behave as protein chaperones (Al-Whaibi 2011). HSPs are involved in protein homeostasis in order to avoid protein misfolding, protein aggregation or protein degradation. They are closely linked with thermo-tolerance and play a crucial role in stress signal transduction (Kregel 2002). In addition to HSPs, plants can produce different osmolytes and antioxidants to protect themselves from various abiotic stresses, including heat stress (Almeselmani et al. 2006); Proline, glycine betaine and aminobutyric acid are key compounds in the osmolyte response under high temperature (Hayat et al. 2012,Chen and Murata 2011,Kinnersley and Turano 2000). Carotenoids, glutathione and ascorbate can act as reactive oxygen species scavengers in order to prevent oxidative stress (Strzalka et al. 2003,Noctor and Foyer 1998,Caverzan et al. 2012). So, the readjustment of cellular homeostasis under heat stress is an essential mechanism which can provide resistance to high temperature and the ability to produce fruit under suboptimal conditions.

In this review, we will focus on the impact of heat stress on the metabolome of pollen (one of the most sensitive organs in plants). We will first review the metabolic changes occurring during pollen development. Secondly, we will describe the impact of heat stress on pollen quality and the metabolic changes associated with thermo-tolerance. Finally, we will discuss various breeding strategies for pollen thermo-tolerance based on the use of metabolic markers.

2. Metabolite Profiles during Pollen Development

Pollen is the male gametophyte and its role is to deliver the genetic material to the embryo sac through the double fertilisation as described by Twell 2002 and Honys et al. 2006. The development of the pollen takes place inside the anthers (Figure 3). In early stages, the anthers have several sporogenous layers. These primary layers lead to the development of meiocytes which occur inside the loculus within the anthers. The loculus is surrounded by the tapetum which provides nutrition, metabolites and enzymes required for the development and the protection of the pollen before it degenerates during mitosis. Meiocytes undergo meiotic division to produce haploid tetrads. The four microspores of the tetrad are released by the action of a glucanase which is produced by the tapetum. At the microspore stage, the pollen coat is synthesised. The pollen wall consists of two layers; an outer layer named the exine, which is composed of sporopollenin and shows apertures where the pollen can germinate; the internal layer is the intine, composed of pectin and cellulose. The pollen coat plays a protective role during pollen dispersion and can also play a role in the attachment to a pollinator. Microspores undergo mitotic divisions to produce the mature pollen, which are composed of a vegetative nucleus and two sperm cells. Between the microspore stage and the mature stage, a phase of vacuolisation occurs which leads to an increase of the pollen size and a polarisation of the nucleus (Pacini et al. 2011). At mature stage, the pollen dehydrates which provides a level of tolerance to environmental stresses (Taylor 1997). Rehydration of the pollen happens on a compatible pistil and leads to germination and growth of the pollen tube inside the pistil to deliver the male gamete for the double fertilisation.

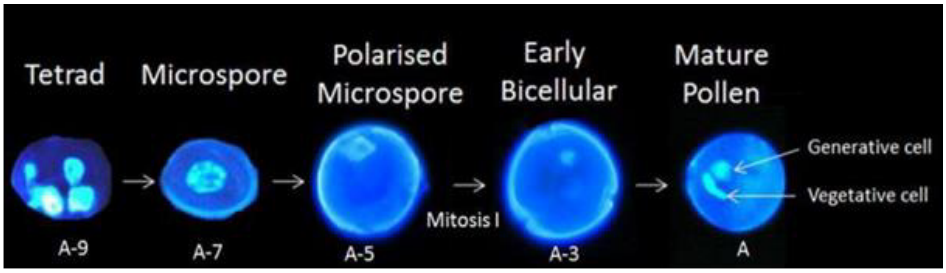

Figure 3. Pollen development from tetrad stage to mature pollen stage. Days before anthesis related to developmental stages are based on *Lycopersicon esculentum* Mill. "Trust" from Sawhney and Bhadula 1988 and Pressman et al. 2002. **A**, anthesis, **A-3**, 3 days before anthesis, **A-5**, 5 days before anthesis, **A-7**, 7 days before anthesis, **A-9**, 9 days before anthesis. Nuclei are stained with DAPI.

2.1. Carbohydrates and Acid Invertase

Carbohydrates are important substrates for plant growth which can be stored as energy reserves and also serve as signalling molecules (Eveland and Jackson 2012). Male sterile lines of Indian mustard showed an altered sugar metabolism in reproductive tissue, suggesting a causal relationship between sugar metabolism and fertility (Banga et al. 1984). Pacini 1996 reviewed carbohydrate reserves in pollen of different species. The main soluble sugars in mature pollen are fructose, glucose, sucrose and starch. Sucrose is produced in photosynthetic tissue such as leaves which is considered as source tissue from which sucrose is transported via the phloem to sink tissue (Roitsch 1999). Pollen is considered a sink tissue which needs sugars for its own development, growth and protection against environmental stresses.

The conversion of sucrose into hexoses, which can be used by the pollen, requires its hydrolysis by acid invertase and/or sucrose synthase (Koch 2004). Koch 2004 reviewed the role of acid invertase in plants; Three different acid invertases can be identified according to their location: vacuolar invertase (VIN), cytoplasmic invertase (CIN) and cell wall invertase (CWIN). The function of acid invertases in reproductive tissues has been intensely studied in different species such as lily (Singh and Knox 1984, Castro and Clement 2007), tobacco (Goetz et al. 2001, Le Roy et al. 2013), tomato (Pressman et al. 2012) and chilli pepper (García et al. 2013).

The physiology of carbohydrate accumulation during anther and pollen development has been studied in lily (*Lilium auratum*) (Clément and Audran 1995, Clement et al. 1996; Clément and Audran 1995), showed that, in lily, the different cells which constitute anthers are connected via plasmodesmata involved in symplasmic transport of carbohydrates from the phloem to the internal anther layers. However, the transport of assimilates from internal anther layers to the pollen follows an apoplastic pathway. The main carbohydrates found in lily anthers were sucrose, glucose, fructose and starch (Clement et al. 1996). During the anther growth, starch was used and this decrease correlated with a strong accumulation of soluble sugars. This was also found by Pressman et al. 2002 in tomato. In addition, Castro and Clement 2007 studied the content of carbohydrates in different fractions of lily anthers, such as anther wall, locular fluid and pollen. The accumulation of soluble sugars in the locular fluid suggested that this tissue

may play a role in sugar storage during pollen development, which is especially useful once the tapetum is degraded. In lily, soluble sugars also accumulated in mature pollen, mainly in the form of hexoses, while in tomato sucrose represented 80% of the carbohydrate fraction in mature pollen (Pressman et al. 2012). Although we cannot exclude that the differential accumulation of hexoses *vs.* sucrose may be species specific, Castro and Clement 2007, notified that the lily pollen used for sugar analyses were hydrated. Using tomato pollen, we observed that hydrated pollen grains have a higher abundance of hexoses than dried pollen grains, due to the action of acid invertase upon (re-)hydration. Therefore, if the pollen isolation protocol requires immersion into a germination solution, we strongly advise to freeze-dry the pollen extract as soon as the harvest is done to prevent the action of acid invertase which may convert sucrose into hexoses, or to incubate the extract in 80% ethanol at 75 °C as it was previously described by Pressman et al. 2012 and Firon et al. 2006 . Nevertheless, Castro and Clement 2007 found a gradient of soluble sugars from the anther to the pollen underlying the strong sink of the pollen. During pollen maturation in tomato, sucrose was the most abundant sugar in the filament and in the pollen grain, whereas hexoses were the most abundant sugars in the anther wall and in the locular fluid (Pressman et al. 2012). The higher abundance of hexoses in the anther wall was supported with a high activity of acid invertase and a low activity of sucrose phosphate synthase. The accumulated hexoses in anther wall could be used for general metabolism or could move into the locular fluid. The distribution of hexoses among the different stamen parts were mainly controlled by cell wall acid invertase. The high accumulation of sucrose in mature pollen could serve as energy source for pollen germination, but could also act as osmolyte to maintain membrane integrity during pollen dehydration (Pressman et al. 2012). To summarize, soluble sugars can be delivered to the pollen by (i) hydrolysis of starch (ii) transport of sucrose from photosynthetic tissues. The sucrose needs to cross several compartments before it reaches the pollen in order to provide nutrition and protection: sucrose has to be transported from the leaves via the phloem to reach the anther wall or directly coming from the anther itself (Clément et al. 1997). Subsequently, it has to cross the locular fluid to reach the male gametophyte. Acid invertases play a major role in supplying hexoses, derived from sucrose, to the pollen grain to support nutrition, growth and protection against environmental stresses.

2.2. Proline

Amino acids play an essential role during plant development: they are the building blocks for protein synthesis. Proline is a free amino acid synthetized from glutamate, which can act as a compatible solute to achieve osmotic adjustment (Lehmann et al. 2010, Szabados and Savoure 2009). Proline is one of the most abundant amino acids in the male reproductive part (Mutters et al. 1989). The changes in amino acid content during pollen development in anthers has been studied in devil's trumpet (*Datura metel)*, a *Solanaceous* species (Sangwan 1978). The content of free amino acids in pollen was higher at mature stage compared to earlier developmental stages. It was suggested that the lower level of amino acids during early developmental stages was due to active protein synthesis at these stages. The accumulation of glutamic acid decreased during pollen development, probably due to conversion into proline. Proline was one of the most abundant amino acids at mature stage and represented 60% of the free amino-acids. In

arabidopsis (*Arabidopsis thaliana*), a proline deficient mutant showed a decreased pollen viability which indicates a role for proline in pollen development. The proline mutant could be partially complemented by spraying proline on the mutant inflorescences. This led to an improvement of pollen germination (Mattioli et al. 2012).

2.3. Lipids

In contrast to carbohydrates, the role of lipids during pollen development has not been studied extensively. Lipids are important compounds for the stabilization of membranes and for the hydration of pollen upon germination (Edlund et al. 2004). Rodriguez-Garcia et al. 2003 showed that lipids accumulated during pollen development in olive (*Olea europaea*). The accumulation of lipids occurred after the vacuolisation of the microspore. During pollen germination, lipids are located close to pollen apertures. This suggests that lipids may act as energy reserve for pollen germination. The lipid profile of pollen at different developmental stages has been studied in trumpet vine (*Campsis radicans*) (Bignoniaceae) (Tütüncü Konyar et al. 2013). Until the microspore mother cell stage, anthers contained low levels of lipids and a high abundance of insoluble polysaccharides. At meiosis, insoluble polysaccharides decreased whereas lipids started to accumulate. The authors suggested that anthers switched from the use of polysaccharides to lipids as storage products. Additionally, lipids have been showed to play a major role in directing the pollen tube to the stigma (Wolters-Arts et al. 1998).

2.4. Gluthatione

Glutathione is a thiol which participates in redox regulation and plays a role in storage, transport, and regulation of metabolites. Furthermore, it is involved in the detoxification of reactive oxygen species (May et al. 1998). The involvement of glutathione in redox regulation plays a crucial role in withstanding environmental stresses (Szalai et al. 2009). The application of buthionine sulfoximine, an enzyme inhibitor in the glutathione pathway, led to a 70% decrease of *in vitro* pollen germination in arabidopsis (Zechmann et al. 2011) demonstrating the crucial role of glutathione in pollen germination.

2.5. Flavonoids

Secondary metabolites produced in the tapetum, such as phenolic compounds, can diffuse to the pollen and play a role in pollen colour, in the attraction of pollinators, in pollen tube germination and in protection against abiotic stress of pollen. Flavonoids can be grouped into different classes, such as flavonols, flavones, isoflavones and anthocyanins. Antioxidant flavonoids play a role in plant development as ROS scavengers and may act as regulators under environmental stress (Agati et al. 2012, Brunetti et al. 2013). Addition of flavonols to germination medium resulted in higher levels of *in vitro* germination of tobacco (*Nicotiana tabacum*) pollen (Ylstra et al. 1992). Down-regulation of chalcone synthase (*CHS*), the first step in the flavonoid pathway, led to a decrease of flavonoids and subsequently to a reduction of pollen germination in petunia (*Petunia hybrida*) and maize (*Zea mays*). The germination could be restored by adding kaempferol to the germination medium (Mo et al. 1992). A confirmation of the importance of flavonoids in pollen fertility has been given by (Napoli et al. 1999) who complemented a petunia *chs* mutant with a functional *CHS* transgene, thereby

restoring the fertility of petunia pollen. In addition, RNAi-mediated silencing of *CHS* in tomato resulted in male sterility, reduced fruit set and seed-less fruit development (Schijlen et al. 2007). In arabidopsis, however, a mutation of CHS (tt4) did not affect pollen germination (Burbulis et al. 1996). The role of flavonoids in pollen fertility is therefore species specific.

2.6. Polyamines

Polyamines, such as putrescine, spermidine and spermine are synthetized from ornithine, arginine or methionine. They play a role in different aspects of plant development such as cell division, embryogenesis, root development, floral initiation, floral and fruit development and also pollen formation (Evans and Malmberg 1989). In tobacco pollen, the polyamine content increased during development from microspore to mature stage, but decreased in germinated pollen, probably due to their consumption during the germination process (Chibi et al. 1993). Song et al. 2001 showed that, despite the decrease of polyamines in germinated pollen, a transient increase of spermidine and spermine at the beginning of pollen tube growth was needed for normal pollen germination in tomato. In the pollen of kiwi (*Actinidia deliciosa*), polyamines were present from early stage (microspore) to mature pollen with spermidine as the most abundant polyamine. Adding polyamine inhibitors led to abnormal pollen development and a reduction in pollen viability, pollen germination and pollen tube growth (Falasca et al. 2010).

2.7. Hormones

Hormones are essential in plant development; they play a role in the regulation of flowering time, leaf senescence, fruit ripening and also pollen development. Parish and Li 2010 underlined the role of auxin, gibberelins and abscisic acid in the development of the tapetum which is essential for the distribution of metabolites to the pollen. In addition, there is accumulating evidence for a role of other hormones, such as ethylene, jasmonic acid and brassinosteroids in pollen development. The current state of the art will be outlined below.

Auxin is involved in many aspects of plant development, such as plant growth, senescence, fruit formation, leaf abscission and apical dominance (Ellis et al. 2005, De Jong et al. 2009, Abeles and Rubinstein 1963, Tanaka et al. 2006). At the genetic level, the biosynthetic pathway to auxin remains unclear and up to five possible pathways have been postulated (Mano and Nemoto 2012). One of the evolutionary most conserved auxin pathways, the indole-3-pyruvic acid (IPA) pathway, is controlled by members of the *YUCCA* flavin monooxygenase gene family. Blocking the auxin biosynthesis pathway in arabidopsis by various combinations of *yuc* loss-of-function mutants led to severe alterations in floral organ development and a lack of pollen production (Cheng et al. 2006). In addition, mutations in auxin receptor encoding genes, such as *transport inhibitor response 1* (*tir1)* and *auxin signalling F box (afb)* genes, resulted in early maturation of pollen due to pollen release before the filament was completely elongated (Cecchetti et al. 2008, Cecchetti et al. 2013). Cecchetti et al. 2013 also proposed that auxin is involved in the coordination of pollen maturation and anther dehiscence. This is supported by the fact that expression of the indole acetic acid lysine synthetase (*iaal*) gene in anther tapetum of transgenic tobacco plants led to a decrease of auxin levels and a concomitant decrease in *in*

vitro pollen embryogenesis (Yang et al. 1997). Culture medium supplemented with auxin restored pollen embryogenesis in transgenic *iaal* plants.

Gibberellins (GA) are known to act in hypocotyl elongation, floral transition, fruit patterning and plant defence (Daviere and Achard 2013). *ga1-1*, an arabidopsis mutant deficient in gibberellin production resulted in an inhibition of stamen elongation and lack of mature pollen. This phenotype could be restored by adding exogenous gibberellins (Goto and Pharis 1999). The mutants GA-deficient *rpe1* and GA-insensitive *slr1-3* in rice are male sterile. *rpe1* was defective in pollen germination and elongation whereas *slr1-3* affected pollen development (Chhun et al. 2007).

Jasmonic acid plays a role in fruit ripening, seed germination, root growth, resistance to biotic stresses and protein storage (Creelman and Mullet 1997). The role of jasmonic acid in pollen fertility has been studied by McConn and Browse 1996. A mutation in the biosynthesis of jasmonic acid, *defective in anther dehiscence1* (*dad*1), led to an inhibition of pollen release. The *dad*1 mutation also led to a 68% decrease of pollen germination after manual pollen release. Pollen germination could be recovered by addition of jasmonic acid (Ishiguro et al. 2001).

Ethylene is known to be involved in plant development, senescence, fruit maturation and pollen germination (De la torre et al. 2006). An inhibitor of ethylene action (NBD) demonstrated the role of ethylene in pollen development in petunia (Kovaleva et al. 2010). Two ethylene peaks were observed during pollen development, at microspore development stage and at maturation. Exogenous NBD completely inhibited anther development at early developmental stages and delayed anther dehiscence. In tobacco, moreover, an ethylene-receptor mutant showed a delay in anther dehiscence compared to the wild-type plants (Rieu et al. 2003). Ethylene, therefore, plays a role in locule opening.

Abscisic acid is important in seed development, plant growth and in withstanding environmental stresses (Xiong 2003).Frascaroli and Tuberosa 1993 showed that a low level of abscisic acid (ABA) in the germination medium increased the germination of maize pollen whereas a higher level decreased germination. In pomegranate (*Punica granatum*), Yang et al. 2003 also noticed that a high concentration of ABA in the germination medium decreased pollen germination. However, a low exogenous ABA concentration in the germination medium did not improve pollen germination. Nevertheless, inhibition of endogenous ABA biosynthesis led to a decrease of pollen germination and it was concluded that a minimal ABA concentration was needed for pollen germination.

Brassinosteroids are steroid hormones which play a role in plant growth and several mutants of the brassinosteroid pathway show abnormal growth characteristics (Fridman and Savaldi-Goldstein 2013). An arabidopsis mutant, *transient defective exine 1* (*tde1*) is deficient in the formation of the pollen exine, which is essential for protection and propagation of pollen. The mutated gene has a high similarity to a gene involved in the biosynthesis of brassinosteroids and supplementation with brassinosteroids led to restoration of exine layer formation (Ariizumi et al. 2008).

During pollen development, several metabolic changes occur leading to the accumulation of reserves necessary for nutrition and protection of the pollen (Table 1). The pathways leading to the production of carbohydrates, amino acids, phenolic compounds, polyamines, hormones and lipids are interconnected and contribute to metabolic homeostasis required for growth and viability of the pollen (Figure 4). This metabolic equilibrium is sensitive to environmental stresses. The effect of heat stress on pollen development and fertility is likely resulting from an altered metabolic homeostasis, caused by alterations in the levels and composition of the above mentioned metabolites. This will be discussed in detail in the next sections.

Table 1. Overview of the role of different metabolites in pollen development and fertility among different species.

Commun name	Latin name	Metabolites	Implication	References
arabidopsis	*Arabidopsis thaliana*	Proline	Required for pollen germination	(Mattioli et al. 2012)
		Glutathione	Required for pollen germination	(Zechmann et al. 2011)
		Auxin	Required for floral organ development and pollen production	(Cheng et al. 2006)
			Required for pollen maturation and anther dehiscence	(Cecchetti et al. 2013)
		Gibberellin	Required for stamen elongation and pollen maturation	(Goto and Pharis 1999)
		Jasmonic acid	Required for pollen germination	(McConn and Browse 1996,Ishiguro et al. 2001)
		Brassinosteroid	Formation of pollen exine	(Ariizumi et al. 2008)
		Flavonoids	Not required for pollen germination	(Burbulis et al. 1996)
Devil's trumpet	*Datura metel*	Amino acids	Accumulation during pollen development Proline represents 60% of the free amino-acids	(Sangwan 1978)
kiwi	*Actinidia deliciosa*	Polyamines	Required for pollen viability and pollen germination	(Falasca et al. 2010)
lily	*Lilium auratum*	Carbohydrates	Accumulation of soluble sugars during anther development Locular fluid is a sugar storage compartment Gradient of sugars from anther wall to pollen grain	(Clement et al. 1996) (Castro and Clement 2007)
maize	*Zea mays*	Flavonoids	Required for pollen germination	(Mo et al. 1992)
		Abscisic acid	High ABA concentration inhibited *in-vitro* pollen germination	(Frascaroli and Tuberosa 1993)
olive	*Olea europaea*	Lipids	Accumulation of lipids during pollen development Energy reserve for pollen germination	(Rodriguez-Garcia et al. 2003)

petunia	*Petunia hybrida*	Flavonoids	Required for pollen germination	(Mo et al. 1992, Napoli et al. 1999)
		Ethylene	Required in anther development and anther dehiscence	(Kovaleva et al. 2010)
pomegrenate	*Punica granatum*	Abscisic acid	High ABA concentration inhibited *in-vitro* pollen germination	(Yang et al. 2003)
rice	*Oryza sativa*	Gibberellin	Required for pollen germination, stamen elongation and pollen development	(Chhun et al. 2007)
tobacco	*Nicotiana tabacum*	Flavonoids	Improve *in-vitro* pollen germination	(Ylstra et al. 1992)
		Polyamines	Accumulation of polyamines during pollen development	(Chibi et al. 1993)
			Reduction of polyamines during pollen germination	
		Auxin	Required for pollen embryogenesis	(Yang et al. 1997)
		Ethylene	Control anther dehiscence	(Rieu et al. 2003)
tomato	*Solanum lycopersicum*	Carbohydrates	Increase of soluble sugars during pollen and anther development	(Pressman et al. 2002)
			Sucrose represents 80% of total carbohydrates in pollen mature	(Pressman et al. 2012)
		Flavonoids	Required for pollen fertility and fruit set	(Schijlen et al. 2007)
		Polyamines	Accumulation of spermidine and spermine is required for pollen germination	(Song et al. 2001)
trumpet vine	*Campsis radicans*	Lipids	Accumulation of lipids during anther development	(Tütüncü Konyar et al. 2013)

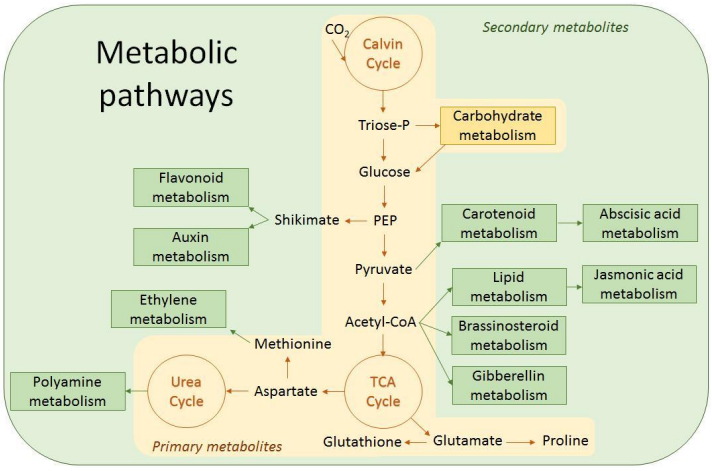

Figure 4. Simplified metabolic pathways underlying the relationship between metabolites reported in this review such as carbohydrates, proline, lipids, glutathione, polyamines, flavonoids and hormones. PEP, phosphoenolpyruvate; TCA, tricarboxylic acid. Triose-P, triose-phosphate.

3. The Impact of Heat Stress on Pollen Quality

3.1. Pollen Viability

Pollen is one of the most sensitive organs to heat stress. This can lead to sterile pollen (Saini et al. 1984) and subsequently a decrease of fruit set (Abdul-Baki and Stommel 1995). As a consequence, crop yield is dependent on the temperature during pollen development (Ploeg Van der and Heuvelink 2005). In tomato, the optimum temperature is between 18 °C and 25 °C (Hurd and Cooper 1970). A few degrees higher than 25 °C already leads to a reduction of yield (Peet et al. 1998). The impact of heat stress on pollen viability has already been demonstrated in several species: tomato (Firon et al. 2006, Pressman et al. 2002, Abdul-Baki and Stommel 1995, Peet et al. 1998, Sato et al. 2006); barley (*Hordeum vulgare*) (Sakata et al. 2000), rice (Prasad et al. 2006, Matsui and Omasa 2002), chickpea (*Cicer arietinum*) (Devasirvatham et al. 2012), maize (Herrero and Johnson 1980), arabidopsis (Kim et al. 2001), rapeseed (*Brassica napus*) (Young et al. 2004), purple false brome (*Brachypodium distachyon*) (Harsant et al. 2013), common bean (*Phaseolus vulgaris*) (Porch and Jahn 2001), groundnut (*Arachis hypogaea*) (Prasad et al. 1999), bell pepper (Capsicum annuum) (Aloni et al. 2001), soybean (*Glycine max*) (Djanaguiraman et al. 2013), and strawberry (*Fragaria x ananassa*) (Ledesma and Sugiyama 2005).

3.2. Pollen Development

At early stages of pollen development, high temperatures lead to an arrest in pollen development (De Storme and Geelen 2014). In tomato, the most sensitive stage is between 10 and 7 days before anthesis (Iwahori 1965). This was confirmed by Sato et al. 2002, who showed that a temperature regime of 32 °C/26 °C, 15–7 days before anthesis, corresponding to the stage in which meiosis takes place, had a profound effect on pollen development. Heat stress during meiosis also has a large effect in barley (Sakata et al. 2000). In cowpea (*Vigna unguiculata*), however, heat stress during meiosis did not affect pollen quality, but when it was too hot after the release of tetrads (between 9 and 7 days before anthesis) an effect on pollen quality was observed (Ahmed et al. 1992). In peanuts, the most sensitive stages were during the development of microspores (four days before anthesis) and at anthesis (Prasad et al. 2001). In bell pepper, heat stress at microspore mother cell meiosis led to reduction of pollen viability, fruit set and seed number, whereas heat stress at later developmental stages did not affect the pollen viability (Erickson and Markhart 2002). The developmental sensitivity to heat stress in pollen seems to be species-specific, with meiosis a common sensitive stage, for most crops.

3.3. Tapetum

The tapetum is the key organ that provides metabolites to the pollen; its development is very sensitive to heat stress. In barley, for example, an elevated temperature of 30 °C led to an early meiotic prophase I and a premature degradation of the tapetum (Oshino et al. 2007). A degeneration of the tapetum under heat stress has also been reported in wheat (Saini et al. 1984),

cowpea (Ahmed et al. 1992), purple false brome (Harsant et al. 2013) and common bean (Suzuki et al. 2001). Asano et al. 2001 showed that heat stress 10 days before anthesis affected the endoplasmic reticulum pattern of the tapetum showing structural abnormalities which subsequently led to a premature degeneration of tapetum. In summary, it is clear that the development of the tapetum is critically sensitive to heat stress and can lead to abnormal anther development. In addition, heat-stress induced abnormalities in tapetum development will also affect the provision of metabolites to the pollen, which can lead to severe effects on pollen nutrition.

3.4. Opening of Loculi

In rice, heat stress affected the opening of the anther loculi which led to a decrease of pollen fertility (Matsui and Omasa 2002). The same result was also observed in tomato (Figure 5). An inhibition of anther loculi opening may therefore block the release of pollen grain.

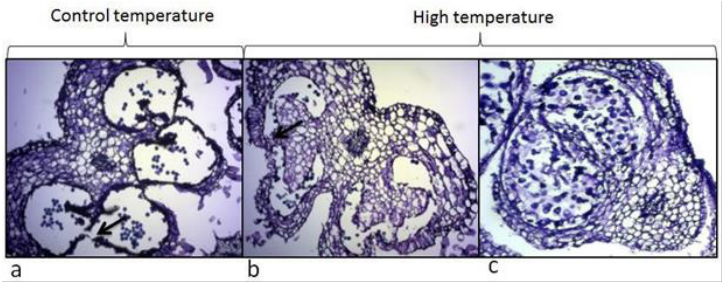

Figure 5. Effect of high temperature (32 °C/26 °C) on anthers of *Solanum lycopersicum* cv. MicroTom. Picture (**a**) shows an anther at mature stage of pollen development under control conditions. The opening of the locule is indicated with an arrow. Picture (**b**) shows anther at mature stage of pollen development under high temperature. The locule was not opened due to anther deformation and the presence of an extra layer of cells. Picture (**c**) shows a severe anther deformation under high temperature. The four distinct locules were no longer visible.

3.5. Pollen Germination

In addition to pollen development, pollen germination is sensitive to heat stress, which can prevent pollen tube growth. Hazra and Ansary 2008 established that the decrease of fruit set of tomato under elevated temperature was mostly due to a reduction of pollen germination. Reduction of pollen germination under heat stress can be due to earlier pollen development failure. High temperatures also led to a decrease of *in vitro* pollen germination in cotton (*Gossypium hirsutum*) (Kakani et al. 2005). The pollen germination starts to decrease at 30 °C (tomato: (Vasil 1987) cucurbita (*Cucurbita pepo*): (Johannsson and Stephenson 1998), cotton: (Kakani et al. 2005)). Pollen germination was altered in tomato under heat stress (Sato et al. 2000) and led to a delay in fruit development and parthenocarpy (Sato et al. 2001, Abdelmageed and Gruda 2009). Plants that can be potentially parthenocarpic become visible when no viable pollen are present; this has been observed in tomato, bell pepper (Aloni et al. 2001, Erickson and Markhart 2002) and in rapeseed (Young et al. 2004).

4. Impact of Heat Stress on Pollen Metabolites

4.1. Carbohydrates

The importance of carbohydrates for plant homeostasis is reflected on the one hand by their function as energy source required for pollen development and germination and, on the other hand, by their role in maintaining osmotic balance, in stress signalling and in protecting membranes. As a consequence, a disruption in carbohydrate metabolism may affect plant nutrition as well as protection against stresses, in both cases leading to a decrease in plant performance and health. The influence of abiotic stresses such as cold, drought and heat on sugar signalling has been recently reviewed (De Storme and Geelen 2014). The impact of heat stress on pollen carbohydrates has been studied in tomato, chickpea, pepper and sorghum (*Sorghum bicolor*) over the last years. In tomato, flowers developed under an elevated temperature of 32 °C showed a decrease of pollen viability. These flowers showed a decrease of soluble sugars in pollen and anther wall, but an increase of soluble sugars in the locular fluid (Pressman et al. 2002). Pressman et al. 2002 suggested that since under high temperature pollen was altered, the pollen's need for soluble sugars was decreased, and therefore soluble sugars were accumulated in the locular fluid. However, we can also speculate that under high temperature soluble sugars are blocked into the locular fluid and cannot reach the pollen. The authors also found that during normal pollen development, starch accumulated in pollen reaching a maximum content three days before anthesis and from this moment onwards starch was converted into soluble sugars. However, under hot conditions, starch concentration did not increase and led to a lack of sugar conversion, explaining the decrease of soluble sugars in mature pollen grains. This indicates that mild chronic heat stress leads to alterations in sugar transport and/or metabolism and an altered distribution of soluble sugars over the different anther tissues. In another study, it was shown that the activity of acid invertase in tomato flowers that were, at 4 days after anthesis, exposed to high temperature (36 °C/28 °C) for 24 h was lower in a sensitive genotype than in a thermo-tolerant genotype. High acid invertase activities may, therefore, play a role in producing hexoses under stress conditions (Li et al. 2011). The effect of heat stress on soluble sugar abundance may be an important factor contributing to the decrease of pollen viability, since thermo-tolerant tomato genotypes, showing high pollen viability under long heat stress exposure (32 °C/26 °C), did not show heat stress-induced sugar alterations compared to thermo-sensitive genotypes (Firon et al. 2006). Alteration of carbohydrate accumulation under high temperature would probably lead to a decreased availability of energy resources and a decrease in the osmotic power of carbohydrates, leading to a failure in pollen development. These results are supported by studies in sorghum in which the decrease of pollen viability under long heat stress (36 °C/26 °C) was mainly correlated with a decrease of starch and sucrose in late stages of pollen development, due to decreased expression of several sugar metabolism genes (Jain et al. 2007). In chickpea, sensitive genotypes had also a lower abundance of sugars in anthers and pollen than tolerant genotypes in a warm season (above 32 °C/20 °C) (Kaushal et al. 2013). The low abundance of sugars was due on one hand to a decrease of sucrose metabolism in leaves and on the other hand to a decrease of sucrose synthase and acid invertase enzymes in anthers. Under high temperature, hexose abundance increased in tolerant genotypes whereas it decreased in sensitive genotypes.

The increase of hexoses may, therefore, have a protective role under high temperatures. A contrasting effect was seen, in bell pepper pollen, where sucrose levels increased rather than decreased when plants were, at eight days before anthesis, exposed to high temperature (32 °C/26 °C) until they reached anthesis. This was mainly due to a decrease of acid invertase and hexokinase concentrations (Aloni et al. 2001, Karni and Aloni 2002). Despite the high levels of sucrose, pollen viability decreased under high temperatures. This suggests that, at least in this bell pepper variety, high levels of sucrose are not sufficient to provide a normal pollen development under high temperature. The contrasting results found in bell pepper compared to the other examples described above could be attributed to the different developmental stages at which the heat stress was applied. In conclusion, activities of acid invertases and the abundance of soluble sugars and starch play an important role in maintaining pollen quality under high temperature.

4.2. Proline

Proline is a common amino acid which can accumulate in response to various environmental stresses. Proline has been shown to play a role in the protection of membrane integrity, in ROS scavenging and in maintaining cellular homeostasis (Hayat et al. 2012, Szabados and Savoure 2009). Incubation of pollen extract in germination medium at 40 °C during 10 min decreased *in vitro* germination of lily pollen and this could be restored by adding proline to the medium (Hong-Qi and Croes 1983). The hypothesis is that proline can stabilise proteins by maintaining the hydration shells around molecules. In cowpea, the content of proline was analysed in anthers of heat sensitive and heat tolerant cultivars developed under high temperature (the maximum temperature per day was 45 °C). The mature pollen of the tolerant cultivars had a higher abundance of proline compared to sensitive pollen, while heat sensitive cultivars accumulated the highest proline levels in anthers (Mutters et al. 1989). The authors concluded that in heat sensitive cultivars the transfer of proline from anthers to pollen was inhibited. The impact of heat stress on proline transport was confirmed by Sato et al. 2006 who showed that the expression of the *proline transporter 1* gene decreased in anthers of tomato under long heat stress (32 °C/26 °C). In contrast, in rice the proline content decreased in anthers of plants exposed to 39 °C for 4 h per day during 5 days. This might be caused by another mechanism involving the transport of proline from the vegetative part to the reproductive part of the plant (Tang et al. 2008a).

4.3. Lipids

Lipids play an important role in membrane fluidity especially under stress conditions. In sorghum, a high temperature of 32 °C/28 °C for 10 days led to an increase of ROS content and membrane alterations in pollen (Prasad and Djanaguiraman 2011). This increase was correlated with a decrease of pollen viability and an alteration in pollen phospholipid content. Under high temperature the abundance of bound unsaturated fatty acids increased whereas the abundance of saturated fatty acids decreased. The authors suggested that membrane damage caused by high temperature could be resulting from the increase of unsaturated fatty acids which will lead to an increase of membrane fluidity due to the presence of double bonds. The unsaturated fatty acid moieties make the membrane more vulnerable to ROS attacks. Similar results were found

in soybean from which the decrease of pollen viability was also correlated to an alteration of in phospholipid saturation (Djanaguiraman et al. 2013).

4.4. Polyamines

Polyamines have been reported to act in tolerance to many stresses such as cold, heat, salt, drought and high metal concentrations (Fariduddin et al. 2013, Gupta et al. 2013). They can act as ROS scavengers and maintain membrane integrity (Alcazar et al. 2006). In tomato, incubation of pollen extract in germination medium for 20 h at 33 °C decreased the *in vitro* pollen germination and this could be reversed by adding spermidine or spermine to the medium (Song et al. 1999). This observation suggested that heat stress decreased the level of polyamines. Indeed, an incubation of pollen at 38 °C during 4 h lowered the content of spermidine and spermine whereas the content of putrescine increased and these changes correlated with a decrease of pollen germination (Song et al. 2002). Adding spermidine or spermine to the medium restored the level of pollen germination. The change in polyamine content and the decrease of pollen germination was mostly due to a decrease of SAMDC (S-adenosylmethionine decarboxylase) under heat stress. Blocking SAMDC translation with cycloheximide could phenocopy the effect of heat stress, leading to decreased pollen germination under control conditions. However, we cannot exclude that other proteins affecting pollen germination may have been inhibited by cycloheximide as well. Contrasting results were found in Japanese apricot (*Prunus mume*), in which a heat stress of 24 h at 35 °C decreased pollen germination, but this could not be restored by adding polyamines to the medium. It was hypothesised that this may have been due to a toxic level of exogenous polyamines (Wolukau et al. 2004). Nevertheless, results in many crops suggest that polyamines have an important role in pollen germination under heat stress.

4.5. Hormones

Brassinosteroids are steroidal hormones which play a role in responses to various abiotic stresses, such as heat, salt and drought stress. They interact with heat shock proteins and also play a role in ROS scavenging (Mazorra 2011). Under heat stress (35 °C for 4 h), the *in vitro* pollen germination of tomato was increased in the presence of 24-epibrassinolide, a brassinosteroid (Singh and Shono 2005), demonstrating a role for brassinosteroids in pollen germination under high temperatures.

Ethylene is also involved in the response to heat stress (Larkindale and Knight 2002). A mutation of the ethylene receptor (*nr*) increased the number of non-viable pollen under long heat stress (32 °C/26 °C) and treatment of the plants with ethylene increased pollen viability under long heat stress (Firon et al. 2012b).

A role for auxin under environmental stresses has been demonstrated in rice under drought conditions (Zhang et al. 2009) and several genes involved in the auxin pathway are known to have a role in withstanding abiotic stress (Jain and Khurana 2009). Under heat stress (30 °C/25 °C for 5 days), the level of auxin decreased in anthers of barley and arabidopsis, which correlated to male sterility (Sakata et al. 2010). The application of exogenous auxin reversed male sterility. Auxin content decreased in anthers of rice plants exposed to 39 °C for 4 h per

day during 5 days and was correlated with a decrease of pollen viability and pollen germination (Tang et al. 2008b). In this same study, the content in abscisic acid (ABA) and gibberelins (GA) was also analysed. GA concentrations decreased under high temperatures and ABA concentrations increased. High levels of ABA were already known to be associated with a decrease of pollen germination (Frascaroli and Tuberosa 1993).

To tolerate heat stress, plants have to maintain metabolic homeostasis in order to avoid disruption of pathways which could lead to abnormal accumulation or reduction of compounds essential for pollen development (Figure 6). A high temperature can affect several compounds at the same time; however, most of the studies are based on the analysis of one group of compounds only (Table 2). Analysis of metabolites in pollen with untargeted approaches could provide a global view on the metabolic responses to heat stress and may lead to the identification of additional, novel compounds involved in the response to heat stress and thermo-tolerance.

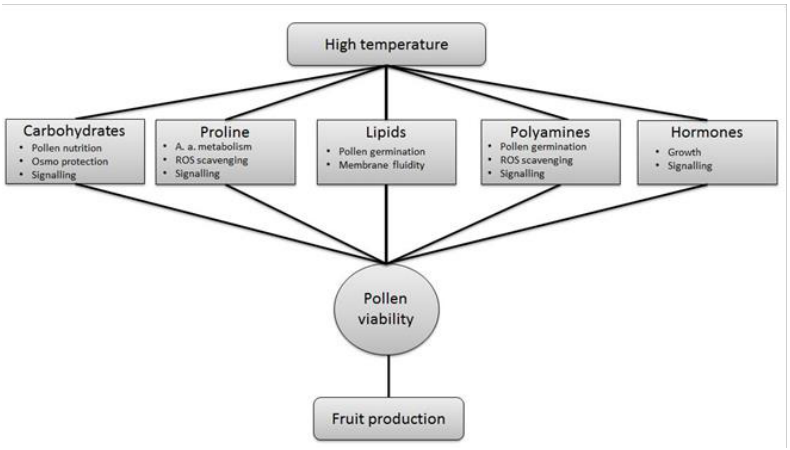

Figure 6. Metabolites affected by heat stress and their role in providing viable pollen.

Table 2. Summary table of the impact of high temperature on the pollen metabolome in different species. HS, heat stress

Common name	Latin name	Metabolites	Temperatures	Heat-stress length	Plant material used	Impacts	Ref
tomato	*S. lycopersicum*	Carbohydrates	32˚C /26˚C	4 months	Flowers totally developed under HS	Decrease in pollen mature	(Pressman et al. 2002)
			32˚C /26˚C	3 months	Flowers totally developed under HS	Sugar abundance decreased in thermo-sensitive genotype compare to thermo-tolerant ones	(Firon et al. 2006)
			36˚C /28˚C	24 hours	Plants at four days after anthesis	Decrease of acid invertase activity in flowers of sensitive genotype compare to tolerant genotype	(Li et al. 2011)
		Proline	32˚C /26˚C	Long	Flowers totally developed under HS	Proline transporter expression decreased in anthers	(Sato et al. 2006)
		Polyamines	33˚C , 35˚C , 38˚C	20 hours	Pollen germination medium	Adding polyamines improved *in-vitro* pollen germination	(Song et al. 2002)
			38˚C	4 h	Pollen germination medium	Decrease in pollen mature	(Song et al. 1999)
		Brassinosteroids	35˚C	4 h	Pollen germination medium	Adding brassinosteroids improved *in-vitro* pollen germination	(Singh and Shono 2005)
		Ethylene	32˚C /26˚C	3 months	Flowers totally developed under HS	Ethylene transporter mutation decreased pollen viability	(Firon et al. 2012b)
barley	*H. vulgare*	Auxin	30˚C /25˚C	5 days	Plants at five leaf stage	Decrease in anther and exogenous auxin restored male sterility	(Sakata et al. 2010)
rice	*O. sativa*	Proline	39˚C	4 h per day during 5 days	Plants at flowering stage	Decrease in anther	(Tang et al. 2008b)
		Auxin	39˚C	4 h per day during 5 days	Plants at flowering stage	Decrease in anther	(Tang et al. 2008b)
		Gibberelin	39˚C	4 h per day during 5 days	Plants at flowering stage	Decrease in anther	(Tang et al. 2008b)

		Abscisic acid	39˚C	4 h per day during 5 days	Plants at flowering stage	Increase in anther	(Tang et al. 2008b)
arabidopsis	*A. thaliana*	Auxin	30˚C /25˚C	5 days	Plants at five leaf stage	Decrease in anther and exogenous auxin restored male sterility	(Sakata et al. 2010)
bell pepper	*C. annuum*	Carbohydrates	32˚C /26˚C	For 8 days before anthesis	Plants at flowering stage	Increase of sucrose due to a decrease of acid invertase activity	(Aloni et al. 2001)
sorghum	*S. bicolor*	Carbohydrates	36˚C /26˚C	Long-season	Flowers totally developed under HS	Decrease of sucrose and starch in pollen mature	(Jain et al. 2007)
		Lipids	32˚C/28˚C	10 days	Plants at 40 days after sowing	Decrease of saturated fatty acids which weaken the membrane to ROS attack	(Prasad and Djanaguirama n 2011)
chickpea	*C. arietinum*	Carbohydrates	Above 32˚C /20˚C	Long	Flowers totally developed under HS	Decrease of sugars in sensitive genotypes compare to tolerant genotype in anthers. Tolerant genotype increased hexose abundance in anthers compare to sensitive genotype.	(Kaushal et al. 2013)
lily	*L. auratum*	Proline	40˚C	10 minutes	Pollen germination medium	Adding proline improved *in-vitro* pollen germination	(Hong-Qi and Croes 1983)
cowpea	*V. unguiculata*	Proline	45˚C /25˚C	Long	Flowers totally developed under HS	Proline accumulated in anther wheareas in pollen it decreased. Tolerant genotype had a higher abundance of proline in pollen	(Mutters et al. 1989)
soybean	*G. max*	Lipids	From 30˚C/23˚C to 39˚C/20˚C	10 days	Flowering stage	Decrease of saturated phospholipids	(Djanaguirama n et al. 2013)
japanese abricot	*P. mume*	Polyamines	35˚C	24 hours	Pollen germination medium	Addind polyamines did not improve pollen germination	(Wolukau et al. 2004)

5. Breeding for Pollen Thermo-Tolerance

5.1. Heritability of Thermo-Tolerance

Since domestication started, humans tried to improve different plant traits such as disease resistance and fruit production, by selecting the most promising plants for the next generation. Nowadays, breeding for a reliable stable yield under environmental stress conditions is an important target for breeders. Global warming has increased breeder's awareness that they should try to improve plants for tolerance to high temperatures. They do that by studying different traits such as fruit set, photosynthesis activity, and pollen quality and by looking for variation in these traits. Rudich et al. 2013 studied thermo-tolerance of different tomato genotypes by analysing fruit set, pollen germination and pollination. Contrasting responses to heat in the diverse genotypes made it possible to identify thermo-tolerant and thermo-sensitive genotypes. It was also shown that the tolerance can be transferred to sensitive genotypes through classical breeding. The heritability of a trait is a measure for a stable inheritance. The heritability depends on the genetic and the environmental variance in a population (Nyquist and Baker 1991). The heritability will be high if the genetic variance plays a relatively big role compared to the environmental variance (Acquaah 2007). For a breeder, a high heritability means that a trait will be stably expressed in the next generations. Several studies on the heritability of thermo-tolerance have been conducted especially in tomato. Hanson et al. 2002 analysed the heritability of thermo-tolerance in an F2 population derived from a cross between a thermo-tolerant and a thermo-sensitive tomato cultivar. They analysed the fruit set and found a low heritability which implies that environmental conditions have a relatively big influence on thermo-tolerance compared to the genetic background of the plant. Alternatively, the low observed heritability may be due to the use of suboptimal, less robust phenotyping methods which reduces the precision of phenotyping and therefore the discriminative power to detect differences. The heritability of different parameters influencing thermo-tolerance, such as pollen viability, pollen germination and fruit set, was studied by (Hazra et al. 2009). They also found low heritabilities for those traits, in line with the results obtained by Hanson et al. 2002. Other studies, carried out with a segregating F3 population of tomato grown in two high temperature environments showed a high heritability for fruit set and fruit weight, but a low heritability for yield (Wessel-Beaver and Scott 1992). The authors concluded that the high heritability of those traits suggested that they can be improved by breeding. However, they also noticed that thermo-tolerance might allow fruit set, but might lead to small fruits. The challenge is to find genotypes with enhanced fruit set and with the capacity to still produce fruits with an acceptable quality. The mechanisms involved in these two processes might be different.

5.2. QTL Mapping for Thermo-Tolerance

Molecular markers are a useful tool for breeders to follow the introgression of specific genomic regions (Quantitative trait loci, QTL) associated with a specific trait (Jain and Brar 2009). The first step is to find QTL. This can be done by using two different forward genetics approaches (Figure 7). The first one is by crossing genotypes that are contrasting for the trait of interest and by producing mapping populations (QTL mapping). The second one is by using an available germplasm collection in which the variation of the trait of interest will be studied (association

mapping). In such populations, associations can be found between molecular markers and the trait of interest. In this way, the QTL analysis can pinpoint specific chromosomal regions on which one or more genes are located that influence the trait. Subsequently, breeders can select for this trait using molecular markers, based on sequence polymorphisms in the region of interest. This is called indirect or marker-assisted selection (Acquaah 2007). Marker assisted selection has already been used to screen wheat varieties with markers related to grain filling duration under heat stress (Sadat et al. 2013). A marker associated with pollen thermo-tolerance could also be used to introduce thermo-tolerance into a sensitive genotype by classical breeding (Figure 7). QTL analyses for thermo-tolerance traits have been carried out (i) by measuring pollen viability, in maize (Frova and Sari-Gorla 1994), rice (Xiao et al. 2011) and tomato (Kardivel 2010), or (ii) by measuring fruit set, in tomato (Grilli et al. 2007, Lin et al. 2010) and in rice (Ye et al. 2012, Jagadish et al. 2010b). QTL identification can be the starting point of more detailed analyses, for example QTL fine-mapping, which might lead to the identification of more closely-linked molecular markers which can be used by breeders to screen at the seedling stage for thermo-tolerant genotypes without too much linkage drag. Eventually, fine mapping can even lead to the isolation of the key genes underlying thermo-tolerance. In cowpea, QTLs related to thermo-tolerance have been identified by determining the number of pods per peduncle in a population of recombinant inbred lines (Lucas et al. 2012). This led to the identification of several candidate genes encoding a heat shock protein, a heat shock transcription factor and a proline transporter. The confirmation of candidate genes can be done in two different, so called, reverse genetics approaches (Figure 7). The first one is by targeting a specific gene in a tilling (targeting induced local lesions in genomes) population and analyse its phenotype. A tilling population is made by randomly mutagenizing seeds (Colbert et al. 2001). The second approach is by producing transgenic lines in which the candidate gene can be (i) silenced using for example an anti-sense construct or (ii) over-expressed using for example a constitutive or tissue-specific promoter. The phenotypic analysis of such lines may confirm that the candidate gene is indeed responsible for the variation of the trait of interest among the population. For example, if a candidate gene involved in pollen thermo-tolerance is over-expressed, a better performance under high temperature is expected compared to the wild-type.

In addition to pollen viability or fruit set, metabolite profiles vary under different temperatures as well, particularly in pollen. The variation in levels of specific metabolites associated with thermo-tolerance can be mapped and used as an alternative way of phenotyping pollen thermo-tolerance. Firon et al. 2006 showed that the sugar levels are maintained in pollen of thermo-tolerant genotypes whereas in sensitive genotypes the sugar levels decreased. It was suggested that sugar level could be a good parameter for thermo-tolerance since it can be easily measured in a more accurate way than pollen viability. The identification of metabolite QTLs (mQTL) associated with thermo-tolerance could therefore be a useful tool to get novel insight into the mechanisms conferring thermo-tolerance (Arbona et al. 2013, Fernie and Schauer 2009). In this review, several compounds that play a role in pollen thermo-tolerance, such as sugars, polyamines and hormones have been described. By studying the segregation of such metabolites in a mapping population, specific chromosomal regions can be identified which may be associated with pollen thermo-tolerance. The complex inheritance, low heritability and

difficult phenotyping of thermo-tolerance make this a difficult trait to improve. The development of a good mapping population and easy and reliable phenotyping methods is the key to find thermo-tolerant QTLs and predictive molecular markers. QTL analysis is not the only way to identify candidate genes related to pollen thermo-tolerance Alternatively, detailed knowledge regarding the trait of interest may pinpoint towards specific candidate genes, whose role in pollen thermo-tolerance can be analysed through reverse genetics approaches in which the candidate gene is over-expressed or down-regulated by transgenic or tilling strategies (Figure 7). For example, in this review different metabolites have been listed for their role in pollen thermo-tolerance, such as for example flavonoids. Over-expression and/or down-regulation of genes involved in the flavonoid pathway could lead to the identification of key genes playing a role in thermo-tolerance. Such genes could form the basis to find (in diversity screens) or create (through tilling) novel genetic variation that can be used to breed for pollen thermo-tolerance, using the candidate gene as marker.

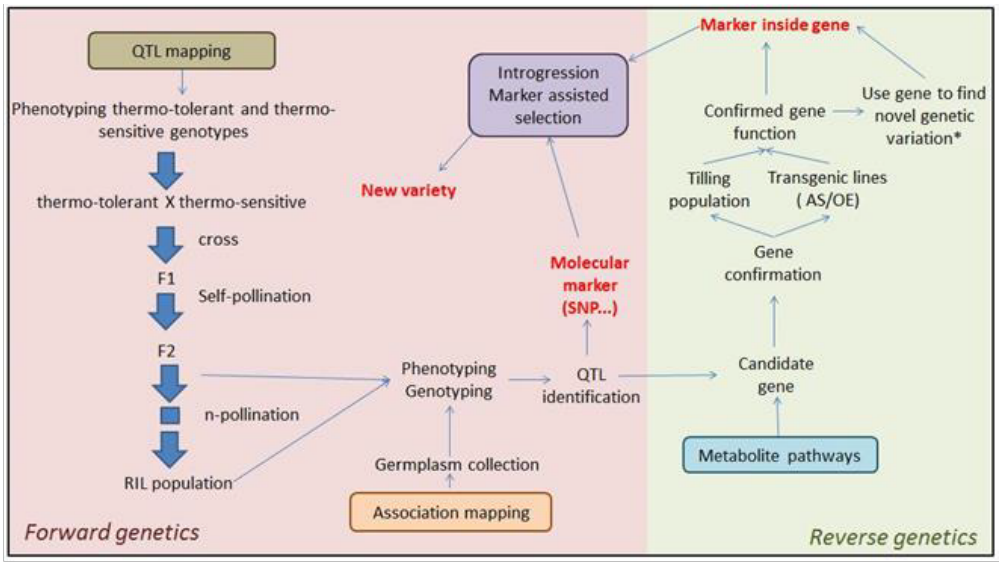

Figure 7. Breeding approaches to improve crop thermo-tolerance and create new thermo-tolerant varieties. QTL, quantitative trait loci; RIL, recombinant inbred lines; SNP, single-nucleotide polymorphism; AS, anti-sense; OE, over-expression. * this approach is only used in the case of reverse genetics

6. Conclusions

Pollens are very sensitive to heat and can be affected by heat stress during different developmental stages. Thermo-tolerant and thermo-sensitive genotypes respond differently to heat stress. This is reflected in differences in metabolite accumulation profiles during development of mature fertile pollen. This review aimed to summarize the current state of the art in our understanding of the metabolic basis for pollen thermo-tolerance. To date, several metabolites have already been shown to play a role in pollen thermo-tolerance. The availability of efficient "non-targeted" metabolic screening methods make it possible to obtain a broad view on the metabolic processes involved in pollen thermo-tolerance and may lead to the

identification of metabolic markers for pollen thermo-tolerance. This will allow a more efficient screening for pollen thermo-tolerance in diverse germplasm and available mapping populations and may lead to the identification of genetic markers and key genes involved in thermo-tolerance. These can be used in breeding programs aimed at improving this important trait in crop plants.

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Chapter 3

The effect of isolation methods of tomato pollen on the results of metabolic profiling

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Abstract

Untargeted metabolomics analysis powerful tool to detect hundreds of metabolites within a given tissue. However, metabolomics approaches are not well developed in the pollen research field. Common pollen isolation procedures require the use of solution to isolate the pollen that is tightly enclosed within the anthers of the flower. These isolation protocols raise a number of concerns for their suitability to metabolomics analyses, including metabolic activity upon rehydration and anther contamination. We assessed the effect of different sample preparation procedures currently used for pollen isolation for their suitability to perform untargeted metabolomics analyses of tomato pollen. Our results demonstrated that (i) pollen isolation with a watery solution led to rehydration of mature pollen grains. This induced metabolic changes in flavonoids, phenylpropanoids and amino acids and a metabolite profile that does not reflect the real biological state of mature pollen, (ii) conversion of sucrose into hexoses took place during the metabolic extraction of rehydrated pollen, but this could be prevented by freeze drying rehydrated pollen and (ii) the presence of anther tissue during the pollen isolation led to contamination of specific metabolites (i.e. alkaloids), most likely originated from anther walls. These compromise the metabolic purity of the pollen fraction. We concluded that the current method used to isolate pollen is suboptimal and give recommendations to improve the pollen isolation protocol, in order to obtain the most reliable metabolic profile from pollen tissue.

Key words : Pollen, metabolome, metabolomic, metabolite, anther, tomato

Introduction

Over the last two decades new technologies have arisen which allow the examination of hundreds to thousands of molecules within a single analysis. Those breakthrough technologies called "Omics" have changed the way research is performed and led to a more comprehensive view of biochemical processes. Metabolomics platforms are used to measure the relative abundance of a large range of metabolites in any given tissue. More than 200,000 different metabolites are estimated to exist in the plant kingdom (Fiehn, 2002). A proportion of these metabolites are specific to a certain tissue, a species, or a condition and this expands the use of metabolomics analyses as a deep chemical phenotyping tool (Fritsche-Neto et al. 2015). For instance, associations between genotype and metabolic phenotype have been studied to identify related markers to for instance fruit and vegetable quality, and biotic and abiotic stress resistance (Tikunov et al. 2005, Riedelsheimer et al. 2012, Wahyuni et al. 2013, Viquez-Zamora et al. 2014 and Xu et al. 2013). Metabolomics analyses generally include the following five major steps: (i) sample preparation, (ii) sample analysis, preferably using different analytical platforms, such as Gas Chromatography and Liquid Chromatography coupled to Mass Spectrometry (GC-MS and LC-MS, respectively), (iii) data processing, including peak picking, peak alignment and compound annotation, as far as is possible, (iv) statistical analysis and (v) data interpretation (Fiehn 2002).

In general, in order to compare the abundance of any metabolite in a set of biological samples it is essential to accurately determine the amount of sample and use the same sample weight per volume of extraction solvent. Therefore, usually between 10 mg dry weight up to 1 g of fresh material, well powdered in liquid nitrogen, is used for a single analysis (De Vos et al. 2007). However, when working with small plant organs or tissues, such as pollen grains or trichomes, it can be highly challenging to obtain this amount of material and determine its exact weight. For instance, the diameter of one pollen grain from tomato (*Solanum lycopersicum*), is only about 20 μm and pollen of at least 10 mature flowers are needed to obtain 4 mg of (dry) tomato pollen.

In plants, pollen is a key organ for a successful reproduction. Its role is to deliver the two sperm cells to the female gametophyte (Twell 2002). The development of mature and fertile pollen grains is required to accomplish its fate, fertilisation of an ovule. Due to the sensitivity of pollen to environmental stresses, which can lead to an alteration of pollen quality and therefore a decrease of yield in many crops, the number of studies on pollen is rapidly expanding (e.g. rice (Saragih et al. 2013), sorghum (Prasad and Djanaguiraman 2011) and tomato (Firon et al. 2006)). To understand the dynamic processes and key steps leading to the development of a mature and fertile pollen grain, several studies focussed on the analysis of transcripts, proteins and metabolites in pollen (Rutley and Twell 2015, Muschietti et al. 1994, Paupière et al. 2014). However, comprehensive metabolomics of pollen grains, such as large-scale untargeted LCMS or GCMS approaches, is not well developed compared to other omics technologies (figure 1). So far, several studies have applied targeted analytical approaches to study specific classes of compounds, such as carbohydrates and polyamines, during pollen development or in relation to environmental stresses, such as high temperature (Pressman et al. 2002, Falasca et al. 2010 and Firon et al. 2006). The extension of such targeted analyses with comprehensive metabolomics

approaches will contribute to a better understanding of the metabolic dynamics occurring in pollen during its development and in response to environmental stresses.

The key to obtain reliable metabolic information of pollen is the development and implementation of reliable methods and protocols for pollen sample preparation and metabolite extraction. Available protocols for pollen isolation raise a number of concerns with regard to the metabolome, that needs to be addressed in order to ensure their suitability for untargeted metabolomics analyses and interpretation of data. Firstly, pollen is commonly isolated by disclosing the anthers in a solution, which also allows the release of unripe pollen in order to study pollen at different developmental stages (Honys and Twell 2004, (Chaturvedi et al. 2013, Firon et al. 2006, Aouali et al. 2001, Castro and Clement 2007). This solution consists of water supplemented with mannitol or salts to prevent bursting of the pollen during their isolation (e.g. Honys and Twell 2004 and Firon et al. 2006). However, during their maturation the pollen grains dry within the anthers (Firon et al. 2012a). Thus, upon extraction from the anthers using a watery solution, the pollen may partially or fully rehydrate. This could lead to unwanted activation of enzymes present in the pollen which consequently may affect the metabolic content and, thus, this pollen isolated in water may no longer reflect its dry mature state. Secondly, to release pollen from the stamen, anthers are cut and/or squeezed (Chaturvedi et al. 2013, Firon et al. 2006). Although filters and washing steps are often used to prevent contamination from the surrounding tissue, these measures cannot fully prevent the release of metabolites from the anthers into the isolation solution. Appropriate measures should be taken to avoid these unwanted artefacts.

Although a great progress has been made in developing complex analytical instruments and powerful data analysis methods for metabolomics, so far little attention has been paid to the impact of the very first steps of any metabolomics experiment on pollen: the sample collection. The objective of this study was to assess the effect of different sample preparation procedures, currently used for pollen isolation, for their suitability to perform untargeted metabolomics analyses of tomato pollen. We used three liquid chromatography-based analytical platforms, HPLC-LTQ Orbitrap MS of aqueous-methanol extract, HPLC-Q Exactive Orbitrap MS of polar extracts and Dionex HPLC-ECD with electrochemical detection, to detect semi polar compounds, organic acids/amino acids, and sugars, respectively. Based on the metabolomics profiles obtained, we provide insight into the benefits, limitations and pitfalls of various approaches for pollen isolation, lyophilisation and metabolite extraction. We provide recommendations to limit metabolic alterations during the whole process of sample preparation and analysis. The present results are not only relevant for future metabolomics studies on pollen, but also provide more insight into the biochemistry of pollen during methods currently used to harvest the pollen for other analytical approaches, including targeted metabolite analyses, proteomics and transcriptomics.

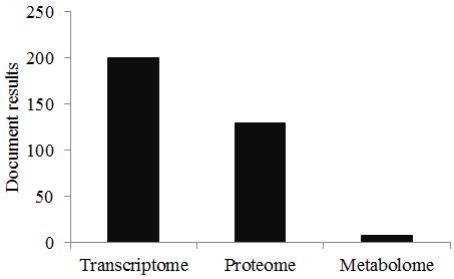

Figure 1. Numbers of documented results from the search on-omics and pollen. The search was performed on Scopus data base (www.scopus.com) with "pollen transcriptome", "pollen proteome" and "pollen metabolome"

Material and methods

Plant materials and growth conditions

Seeds of the tomato (*S. lycopersicum*) genotype M-82 were obtained from the Tomato Genetics Resource Centre. M-82 plants were grown in the greenhouse of Wageningen University & Research Centre (The Netherlands) at 25˚C during the day and 19˚C during the night under approximatively 13 hours of natural day light.

Pollen isolation methods

Two pollen isolation methods were applied: (i) dry isolation by flower vibration, and (ii) wet isolation by anther squeezing in isolation solution (figure 2).

For the flower vibration method (i), pollen were directly isolated by vibrating the flower still attached to the plant, using a milk frother to let the dry mature pollen fall into a 1.5-mL Eppendorf tube. This method was used in most of the experiments, in particular for the samples isolated by (a) vibration and then directly lyophilised (VL) and the technical replicates of these VL pollen (VLT), (b) vibration-derived pollen incubated in isolation solution before lyophilisation (VS), and (c) vibration-derived pollen incubated in isolation solution and then lyophilised (VSL). For the squeezing method (ii), the flowers were detached from the plants and kept on a petri dish on ice before pollen isolation. This method was used for pollen samples isolated by squeezing in solution and then lyophilised (AL). An overview of the different samples used are shown in figure 2 and in table 1. VL and VSL samples were used to assess the metabolic changes by the presence of ice cold isolation solution. VSL and VS samples were used to compare the impact of lyophilisation. AL and VSL samples were used to analyse metabolite contamination from the anthers. VLT samples were used to determine the technical reproducibility of the metabolite extraction and metabolomics profiling of exactly the same pollen material. For VL and VS six biological replicates were used, whereas for AL and VSL five biological replicates were used. Each biological replicate consisted of a pool of approximatively 10 flowers from a single plant, resulting in the isolation of 4-8mg of mature pollen. For VLT samples, the pollen of six plants were pooled and subsequently divided into six aliquots for metabolite extraction.

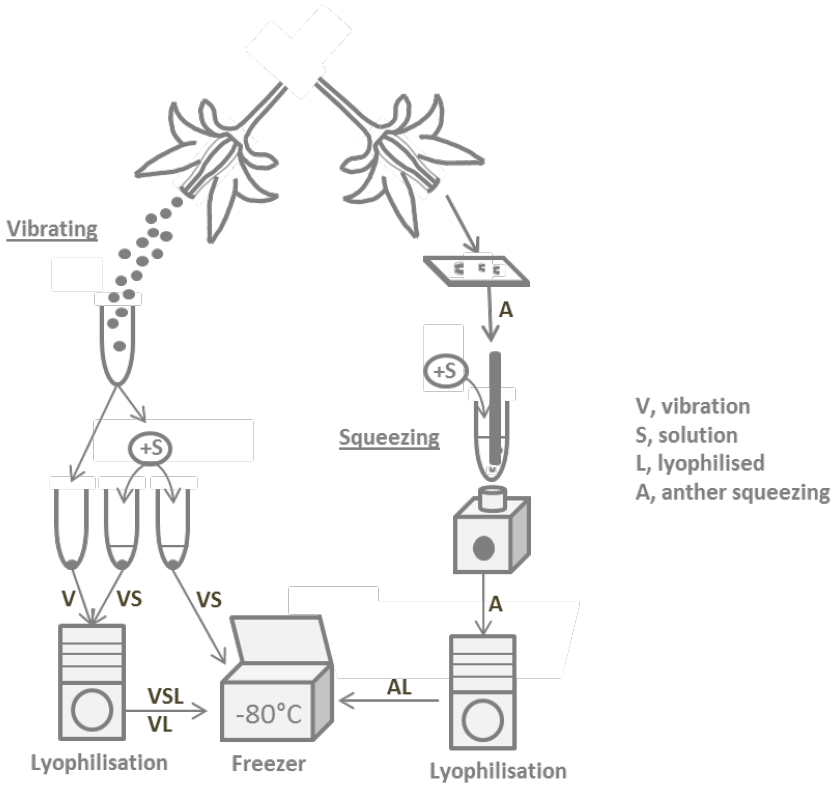

Figure 2. Schema of pollen isolation methods tested. VSL: sample isolated by vibration incubated for one hour in germination solution (+S) and lyophilised; VL: sample isolated by vibration and lyophilised; VS: sample isolated by vibration incubated for one hour in germination solution and non lyophilised; AL: sample isolated by anther (A) squeezing in germination solution and lyophilised

The isolation solution used to isolate pollen is described by Firon et al. 2006. The solution consisted of 1 mM KNO_3, 3 mM $Ca(NO_3)_2$, 0.8 mM $MgSO_4$ and 1.6 mM H_3BO_3 in distilled water. In both VSL and VS samples, 500 µL of ice cold solution was added to the 1.5-mL Eppendorf tube containing the mature pollen, followed by vortexing for one minute and then keeping on ice for one hour, corresponding to the approximate time necessary to isolate pollen in a standard experiment (data not shown). Eppendorf tubes were then centrifuged at minimum speed of 100 g for 2 minutes at 4˚C, followed by short spin at maximum speed of 17,000 g to spin down the pollen as a pellet. 400 µL of the supernatant was removed by pipetting and the remaining sample was frozen in liquid nitrogen and stored at -80˚C.

For AL samples, pollen was isolated using an adapted version of the protocol described by (Firon et al. 2006). In short, sepals and pistil were removed from the flower, after which the anther cone was cut into three pieces with a sharp razor blade and transferred into a 50-mL falcon tube on ice. Then, 10 mL of ice cold solution was added and the anthers were gently squeezed against the falcon tube wall with a 13-mL Sarstedt tube before to be vortexed for ten seconds to allow pollen release. The liquid was filtered with two layers of miracloth

Calbiochem® and transferred into a clean 50-mL falcon tube on ice. The sample was centrifuged at a minimum speed of 8 g for 15 minutes at 4˚C. During the centrifugation, the layers of miracloth were kept in the top of the tube enclosed, in order to recover the solution present in the miracloth. The supernatant was discarded and the pollen pellet was washed again with 500 µL of ice cold solution to limit anther contamination. The sample was shortly vortexed and the pollen suspension was transferred into a new 1.5-mL Eppendorf tube which was centrifuged at minimum speed of 100 g for 2 minutes at 4˚C followed by short spin. 400 µL of supernatant was removed by pipetting and samples frozen in liquid nitrogen and stored in -80˚C.

After preparation, all samples were frozen in liquid nitrogen and stored at -80˚C until metabolite extraction. To obtain lyophilized samples, the material was subjected to lyophilisation for 72 hours.

Homogenizing of pollen material

Flowers of tomato were used to test different pollen homogenization methods for metabolite extraction. Pollen isolated by the squeezing method and lyophilized for 72 hours were used. Two different methods were used to grind pollen: (i) manual grinding with a pestle (pestle samples) and (ii) mechanical grinding with a tissue-lyser II Qiagen. Lyophilised mature pollen were used for each condition. For the pestle samples, pollen were ground with a polypropylene Eppendorf micro pestle in liquid nitrogen, the pollen powder was transferred in a 1.5-mL Eppendorf tube. 210 µL of 100% methanol and 90 µL of distilled water were added to the 1.5-mL Eppendorf tube to reach a final concentration of 70% methanol. For the tissue-lyser samples, methanol was added similarly as described above and three 2-mm stainless steel beads were added to the Eppendorf tubes. Pollen were subsequently ground with a tissuelyser for 5 minutes, 10 minutes or 15 minutes, with a frequency of 25 Hz. From each homogenized extract a 10 µL aliquot was loaded into a Fuchs-Rosenthal haemocytometer (W. Schreck Hofheim/Ts) to count pollen grains that were still intact. Extracts were inspected under a light microscope and pollen grains were scored as still intact when they had a round shape with no visible bursts.

Metabolite extraction for VL, VLT, VSL and AL samples

The preparation of both polar and semi-polar metabolite extracts from pollen materials was carried out at room temperature using a protocol adapted from Wahyuni et al. 2013 and Carreno-Quintero et al. 2012. Before the homogenization step described above, a 10 µL aliquot of the aqueous-methanol extract was transferred into a new 1.5-mL Eppendorf tube containing 500 µL of isolation solution for pollen counting. The pollen counting was performed as described above and the pollen number served to normalise the metabolic data. The presence of 70% methanol did not disrupt the pollen cells, which allowed the pollen counting. Then, pollen homogenization was performed as described above with a tissue lyser grinding for 15 minutes at 25 Hz. The ground sample was sonicated for ten minutes and centrifuged at maximum speed of 17,000 g for ten minutes. Then 200 µL of the methanol supernatant was transferred into a new 1.5-mL Eppendorf tube containing 200 µL of 70% methanol to avoid metabolite saturation. The extract was then filtered over a 0.2 µm polytetrafluoroethylene

(PFTE) filter. The filtered extract was divided in three parts for LTQ Orbitrap LC-MS, Q-exactive LC-MS and HPLC-ECD analysis, respectively.

For the LTQ orbitrap LC-MS, 100 µL of filtered extract was transferred into a 2-mL crimp glass vial with insert and directly used for analysis.

For both the Q-exactive LC-MS and the HPLC-ECD, 80 µL of filtered extract was transferred into a new 1.5-mL Eppendorf tube. Then, 80 µL of distilled water and 40 µL of chloroform were added and the sample was mixed for five minutes followed by centrifugation at maximum speed of 17,000 g for ten minutes. 100 µL of the supernatant was transferred into a new 1.5-mL Eppendorf tube for HPLC-ECD, while 55 µL of supernatant was transferred into a new 1.5-mL Eppendorf tube. Both aqueous-methanol extracts were dried overnight in a speed vacuum.

For the HPLC-ECD, the dried extract was re-suspended in 100 µL of distilled water by pipetting and vortexing. Then 100 µL of a ion exchange resin was added (van Arkel et al. 2012). The sample was mixed on a roller band at 1,000 rpm for five minutes at room temperature. The mixture was centrifuged at maximum speed of 17,000 g for five minutes and 100 µL of supernatant was transferred into a HPLC vial.

For the Q-exactive LC-MS, the dried methanolic extract was re-suspended in 55 µL of distilled water by pipetting and vortexing. Then 50 µL was transferred into a 2-mL crimp glass vial with a 100 µL insert.

Metabolic profiling

The LTQ Orbitrap LC-MS system was composed of a C18 column (phenomenex), a Water Acquity HPLC connected to a photodiode array (PDA) detector and an LTQ/Orbitrap hybrid mass spectrometer as previously described by van der Hooft et al. 2012 and by Moco et al. 2006. The ion source was set in negative ionization ion mode. For the measurements, 10 µL of sample was injected into the system. The Xcalibur program was used for data collection.

The Q Exactive Orbitrap LC-MS analyses were carried out with a Dionex Ultimate 3000 Series RS pump coupled with a TCC-3000RS column compartment and a WPS-3000RS auto sampler (Thermo Fisher Scientific, Waltham, MA). A Discovery HS F5-3 (Supelco: 150*2.1 mm, 3 um particles) column was used for chromatographic separation at 40 °C. Mobile phase A consisted of water and mobile phase B of acetonitrile, both acidified with 0.1% formic acid. The gradient started with 0% B for 5 min and was increased from 0% to 25 % in 20 min. Then the column was washed by increasing mobile phase B to 80% in 5 min and held constant for 3 min. Finally, the mobile phase returned to 0% B and maintained for 8 min to equilibrate the column. A flow rate of 0.1 mL/min and an injection volume of 5 µL was used. The detection of compounds was performed using a Q-Exactive Plus mass spectrometer (Thermo Scientific). A heated electrospray ionization source (HESI-II) in positive/negative ionization mode switching was used for ionization. The ionization voltage was optimized at 3.5 kV for positive mode and 2.5 kV for negative mode; capillary temperature was set at 250 °C; the auxiliary gas heater temperature was set to 220 °C; sheath gas, auxiliary gas and the sweep gas flow were optimized at 36, 10 and 1 arbitrary units, respectively. Full scan data in both positive and negative mode

was acquired at a resolving power of 35,000 FWHM at *m/z* 200. A scan range of *m/z* 90-1350 was chosen. The automatic gain control was set at 3e6 and the injection time was set to 200 ms. External mass calibration was performed in positive and negative modes before each sample series. The Xcalibur software was used for data collection.

The HPLC-ECD (Dionex) was composed of a Carbopac™ PA-100 guard column, a Carbopac™ PA-100 (4 × 250) column and an ED40 Electrochemical detector (Dionex). The sugar elution was previously described by (van Arkel et al. 2012).

Metabolomics data processing

For the LQT Orbitrap LC-MS, data were processed using MetAlign software (available from www.metalign.nl; (Lommen 2009), to correct for baseline, peak picking, and mass alignment of chromatograms as previously described by Tikunov et al. 2005 and De Vos et al. 2007, respectively. The mass peaks were extracted and aligned by MetAlign sofware at a signal cut-off threshold setting of 12,500 ion counts per scan. Processed mass signals were kept for further analysis when they were present in at least all the biological replicates per treatment. MSClust software (Tikunov et al. 2012) was then used to group mass features originating from the same molecule and to extract quantitative ions of compounds represented by the highest intensity ion within the feature group. If a quantitative ion selected by MSClust showed saturation of the MS detector, this ion was replaced by its second or third isotopic ion. Putative annotation of metabolites detected was performed using an in-house accurate mass/retention time database generated by previous LCMS experiments on tomato tissues (Moco et al. 2006; van der Hooft et al. 2012) and the online database METLIN (http://metlin.scripps.edu/). The level of identification was performed according to The Metabolomics Standards Initiative requirements (Sumner et al. 2007): identified compounds got level I when Nuclear Magnetic Resonance was previously performed or an authentic standard has been used for unambiguous identification, level II when no authentic standard was used but annotation was made with both physicochemical property and spectral similarities, level III when the (class of the) compound has previously been reported for tomato, and finally level IV in case further annotation of the detected metabolite was impossible.

For the Q exactive LC-MS authentic standards of the organic acids malic acid, succinic acid and the amino acids proline, serine, asparagine, glutamine, threonine, glutamate, lysine, histidine, arginine, GABA, valine, isoleucine, leucine, and phenylalanine were used to ensure unambiguous identification. Chromatograms were extracted with Xcalibur software. A processing method was established using a ICIS peak integration based on the authentic standards that considered the mass (*m/z*) at 5 ppm window, the retention time, the smoothing points, baseline window, area noise factor and peak noise factor. The parameters were optimized for each detected metabolite. The height of the peak was then retrieved from the peak integration file.

For the HPLC-ECD (Dionex), sugars were identified and quantified (in mg/ml) using a calibration curve of authentic fructose, glucose and sucrose standards.

Statistical analysis

We used between five and six biological replicates for VL, VSL, VS and AL samples and six technical replicates for TL samples. All the metabolites were normalised by pollen number which varied between 156 and 56. To determine which metabolites were different between conditions, a one-way anova per single metabolite was carried out on log2 transformed values using the IBM SPSS statistic software package 20 (www.ibm.com). Performing a large number of statistical tests can lead to an increase of type I errors (false positives). For instance, with a classical significance threshold alpha of 0.05 per single test and the number tests equal to the number of detected metabolites (57), 0.05*57 ≈ 3 metabolites could be expected to be false positives when no real differences exist. Hence, due to this high number of variables, we used a lower significance threshold alpha of 0.005. Metabolites showing significance in the one-way anova test were followed up using a Tukey's HSD post hoc test (alpha=0.05).

Results

In the procedure from pollen isolation to the final analysis of analytical data, the following steps were distinguished and examined:

a) Pollen isolation and release
b) Lyophilisation
c) Pollen homogenisation
d) Technical reproducibility

Figure 2 gives a schematic overview of the different steps in this procedure. An overview of the different parameters tested and samples used is shown in table 1. In total, we annotated 56 metabolites including secondary metabolites detected by the HPLC-LTQ Orbitrap MS (Supplementary data table 1), amino/organic acid detected by HPLC-Q Exactive Orbitrap MS (Supplementary data table 2) and sugars by Dionex HPLC (Supplementary data table 3).

Table 1. Overview of samples used in this study

Abb.	Pollen isolation method	Incubation in germination solution	Lypophilisation	Purpose
VLT	Vibration	No	Yes	Reproducibility
VL	Vibration	No	Yes	Rehydration
VS	Vibration	Yes	No	Sugar conversion
VSL	Vibration	Yes	Yes	Contamination Sugar conversion Rehydration
AL	Squeezing	Yes	Yes	Contamination

It is worth mentioning that the possible influence of the resulting differential sample matrix, due to adding salt-containing solution or not, was firstly assessed: from all the compounds detected by the three metabolomic platforms, only malate and arginine showed a decreased

peak intensity in the presence of the salty solution, most likely due to ion suppression caused by solution components that co-eluted with these two metabolites (Data not shown). Therefore, the interpretation of differential pollen isolation effects on these two compounds must be addressed with care. We did not use a Gas Chromatography-Mass Spectrometry platform to detect polar metabolites (e.g. sugars, amino/organic acids), because most of these metabolites showed a decreased derivation efficiency when isolated in solution, due the presence of salts (data not shown). This made it impossible to compare samples isolated with different methods.

Pollen isolation and release methods

Pollen can be isolated by different methods. The most easy and direct manner to isolate pollen was vibrating the flowers, leading to the release of mature dry pollen. However, flower vibration does not allow the isolation of pollen at earlier stages of development, since they are still attached to the anther tissue. Isolation of unripe pollen is therefore usually achieved by cutting anthers of different sizes (i.e. different developmental stages) into pieces with a razor blade and subsequently releasing their pollen by squeezing the anthers in an isotonic solution (Chaturvedi et al. 2013). This method was called “anther squeeze method”. After its isolation, the pollen was lyophilised, if applicable, and stored at -80˚C until further use.

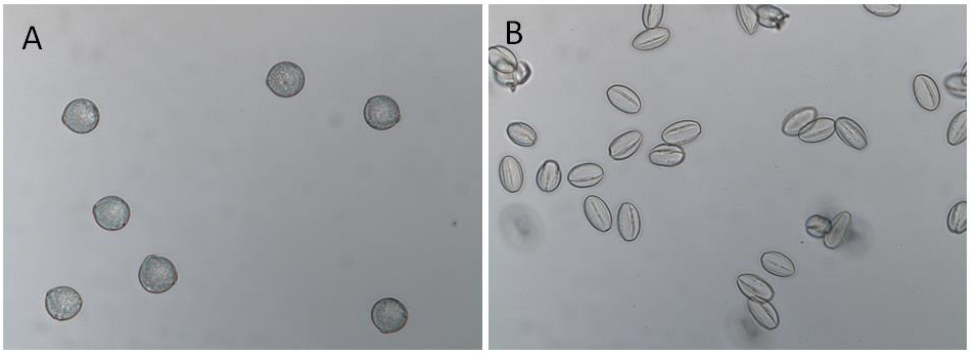

Figure 3. Pollen shape. Pictures of imbibed (A) and dry (B) mature pollen of M-82 observed under a light microscope. Dry pollen was observed in oil to avoid imbibition

Pollen rehydration during isolation

When dry mature pollen was isolated in solution, the pollen underwent a rehydration process which was clearly visible under a light microscope (figure 3). Pollen increased their volume and obtained a round shape. To determine if these morphological transformations was accompanied with metabolic changes, freshly harvested dry mature pollen (VL) were compared with the same pollen incubated for one hour in solution (VSL). To correct for their differential water contents, both samples were freeze-dried before metabolite extraction. Six metabolites, out of the total of 56 annotated showed a significant difference of more than 2-fold between VSL and VL samples (Supplementary data tables 1 and 2). The amino acid glutamate, two flavonoids (i.e. kaempferol aglycone and kaempferol-glucoside-rhamnoside) and the phenolic acid 5-caffeoylquinic acid were 1.8, 2.3, 2.4 and 4.7-fold more abundant in VL samples than in VSL samples, respectively (figure 4A and B), whereas the amino acids serine, glutamine, and

the organic acid malate were 4.7, 2.0 and 3.2-fold more abundant in VSL samples than in VL samples, respectively (figure 4B). This comparison indicates that isolation of mature pollen in solution, as compared to vibration derived pollen, leads to metabolic changes likely due to rehydration, and therefore should be regarded as "imbibed pollen".

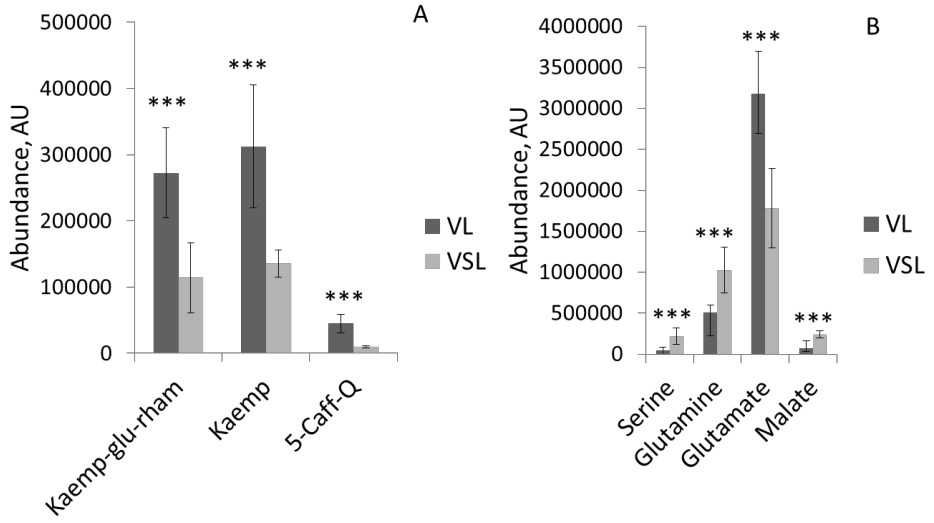

Figure 4. Metabolites affected by rehydration. A, compounds detected by the Orbitrap LC-MS method and B, compound detected by Q-Exactive LC-MS. VL: vibrated pollen directly lyophilised; VSL: vibration derived pollen firstly incubated in germination solution and then lyophilised. Kaemp-glu-rham: Kaempferol 3-alpha-D-glucoside-7-rhamnoside; Kaemp, kaempferol; 5-Caff-Q, 5-Caffeoylquinic acid. Error bars represent the standard deviation of the means (n= 5-6)

Effect of pollen release from anthers

When anthers are squeezed in a solution, contamination by anther tissue may take place (figure 5), but these anther pieces are mainly removed by filtering through miracloth.

Nevertheless, upon squeezing, metabolites from the anther tissues may be dissolved in the isolation solution. We therefore compared the metabolite profile of mature pollen released by squeezing (AL) with that of mature pollen released by vibration followed by a one hour incubation in isolation solution (VSL). Thus, both pollen samples were rehydrated in isolation solution for the exact same time, and were then lyophilised. All sugars, amino acids and organic acids, detected with both the Q exactive LC-MS and the Dionex HPLC system, showed less than 2-fold differences between the two pollen release methods (Supplementary data table 1 and 2). However, more than half of the annotated semi-polar compounds, detected

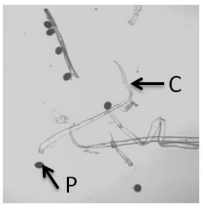

Figure 5. Contamination from anther tissue during pollen isolation. Pollen grains were stained with Alexander dye for a better visualisation under light microscope. P, pollen grain and C, anther contamination

by LTQ orbitrap LC-MS, showed at least a 2-fold difference between the two release methods (Supplementary data table 1, figure 6). Most of the alkaloids present in the AL samples were not detectable in the VSL samples. The same was true for the phenylpropanoid feruloyl quinic acid. In addition, several other compounds were significantly higher in AL than in VSL samples, such as the flavonol glycoside kaempferol-glucoside-rhamnoside (5.1-fold), two forms of caffeoyl-dicoumaroyl spermidine (up to 4.2-fold) and dicoumaroyl spermidine (2-fold) (figure 6B and 6C); the flavonol quercetin-glucoside was detected in VSL samples only.

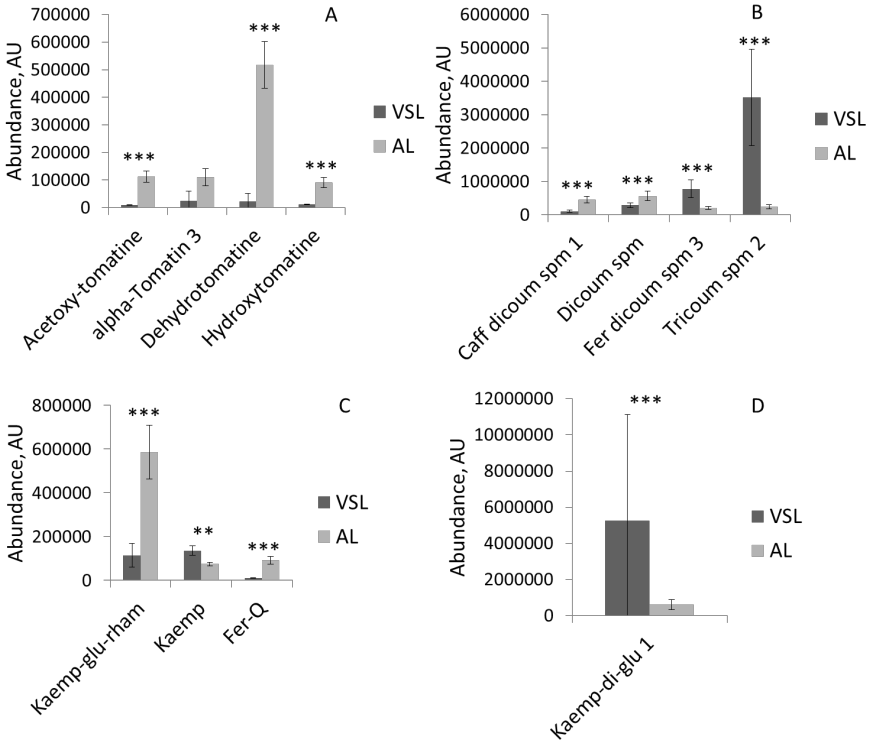

Figure 6. Metabolites affected by the release method. VSL: Vibration derived pollen incubated in germination solution and then lyophilised; AL: pollen isolated by squeezing and then lyophilised. A, alkaloids; B, polyamines; C, flavonoids and phenolic acids; D, flavonoid. caff, caffeoyl; dicoum, dicoumaroyl; spm, spermidine; fer, feruloyl; tricoum, tricoumaroyl. Kaemp, kaempferol aglycone; glu, glucoside; rham, rhamnoside; Q, quinic acid. Error bars represent the standard deviation of the means (n=5-6)

In contrast, the flavonoids kaempferol diglucoside 1 (8.4-fold), kaempferol aglycone (1.8-fold), three forms of the polyamines feruloyl-dicoumaroyl spermidine (up to 25-fold) and tricoumaroyl spermidine (15-fold) showed a lower accumulation in AL samples compared to VSL samples (figure 6B, 6C and 6D). These results clearly indicate that the two different pollen isolation methods lead to samples with a markedly different metabolite composition.

The effect of lyophilisation on the metabolic profile of tomato pollen

Pollen released into isolation solution are rehydrated and can be dried again by lyophilisation (freeze drying), in order to correct for variable pollen water contents, for instance due to differential development stage or germination time. Tomato pollen is known to contain a very active acid invertase that may lead to conversion of sucrose into glucose and fructose during isolation of pollen in solution and subsequent downstream processing (Pressman et al. 2002). To test whether lyophilisation might help in preventing such enzyme-related metabolic conversions, dry pollen was isolated by flower vibration and incubated for one hour in solution, to mimic the isolation in solution with squeezing, and subsequently frozen in liquid nitrogen. Half of the sample was dried by lyophilisation for 72 hours before metabolite extraction (VSL samples), while the other half was extracted "wet" (VS). During tissue homogenisation and metabolite extraction, the ratio solvent/water used for lyophilised (VSL) and non-lyophilised samples (VS) was adjusted to correct for their differential water contents.

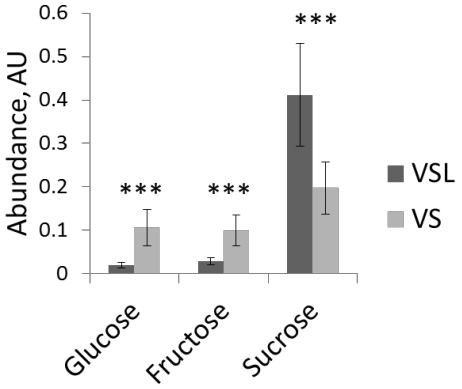

Figure 7. Metabolites affected by lyophilisation. VSL, Vibration derived pollen incubated in germination solution and lyophilised; VS, vibrating pollen incubated in germination solution and non-lyophilised. Error bars represent the standard deviation of the means (n=5-6)

Among the annotated metabolites detected by the three metabolomics platforms, only the three sugars sucrose, fructose and glucose showed a statistically significant and more than 2-fold difference between the two samples (Supplementary data table 3). The hexoses glucose and fructose were present at 5.7 and 3.5-fold higher levels, respectively, whereas sucrose levels were 2-fold lower in VS samples compared to VSL samples (figure 7). We obtained similar results when pollen was isolated with the standard isolation protocol using anther squeezing method (data not shown). These results revealed that lyophilisation of rehydrated mature or wet unripe pollen is an important step to avoid conversion of sucrose into hexose sugars. These

results also suggest that this conversion can still take place after the pollen isolation step, i.e. during their homogenisation and/or extraction.

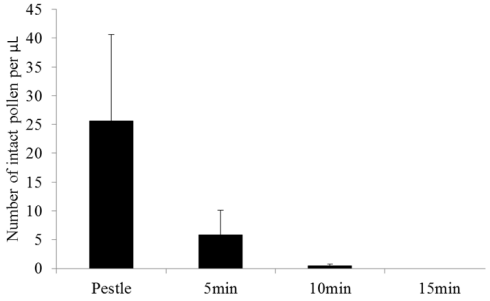

Figure 8. Number of intact pollen grains remaining after different grinding methods. Pestle: manual grinding with an Eppendorf micropestle; 5,10,15min: grinding with three stainless steel beads and an automatic tissuelyser. Number of pollen grains remaining after treatments were counted under a light microscope. Error bars represents the standard deviation of the mean

The effect of pollen homogenization

To determine the optimal method to obtain homogenized pollen material for metabolomics studies, we compared two different homogenization techniques for mature pollen: (ii) manual grinding using an Eppendorf micro pestle and (ii) mechanical grinding using a tissue-lyser. The latter was tested at increasing duration of grinding (figure 8). Subsequent counting of the number of intact pollen under the microscope (figure 8) revealed that the grinding with a micro pestle gave always more intact pollen per µL than with the tissue-lyser: intact pollen were clearly visible in the pestle samples, while grinding with the tissue-lyser for only 5 minutes already broke most pollen and after 15 minutes no intact pollen was observed at all. Thus, the mechanical grinding for 15 min was the optimal homogenizing method for mature pollen.

Technical reproducibility

Dry pollen of different tomato plants was pooled and divided into six aliquots in order to assess the technical reproducibility of metabolite extraction of pollen tissue. In total 50 metabolites could be annotated in these pollen (Supplementary data table 1,2,3). A high correlation coefficient of 0.99 was found in the different aliquots with a Cronbach's alpha coefficient of 0.997 indicating a strong reproducibility between the technical replicates (table 2). Individual coefficients of variation were determined for each compound and 94% of the compounds (47 compounds) showed a coefficient of variation less than 35% (Supplementary data table 4). The glycoalkaloid alpha-tomatin, the flavonoid quercetin 3-O-glucoside and feruloyl-dicoumaroyl spermidine 1 had a coefficient of variation larger than 45%, suggesting that the extraction method used may be further optimized, at least for these three compounds.

Table 2. Coefficient of correlation between the technical replicates based on Person correlation

Correlation Matrix						
Item	VLT1	VLT2	VLT3	VLT4	VLT5	VLT6
VLT1	1.0000					
VLT2	0.9993	1.0000				
VLT3	0.9992	0.9988	1.0000			
VLT4	0.9996	0.9998	0.9988	1.0000		
VLT5	0.9996	0.9998	0.9992	0.9997	1.0000	
VLT6	0.9990	0.9997	0.9981	0.9996	0.9997	1.0000

Cronbach's alpha : 0.997

Discussion

We have shown that diverse aspects of the pollen isolation procedure can influence the final result of the pollen metabolome analysis, independently from the variables under study, such as growing conditions, developmental stage or genetic background. These aspects will be discussed below and recommendations will be given in order to obtain the most reliable metabolic data from pollen.

Imbibed versus dry pollen

During pollen development, the pollen of tomato undergoes a desiccation process in the final stage of its maturation (Firon et al. 2012a), thereby conferring tolerance to abiotic stresses. We observed that the incubation of pollen in solution during isolation led to pollen rehydration. Therefore, pollen isolated in such a way needs to be considered as imbibed pollen (with analogy to seed). Although isolation in a solution is the most widespread method, isolation of dry mature pollen without solution is also found in literature (Song et al. 2001, Song et al. 2002). We called this isolation method flower vibrating with further pollen lyophilisation (VL). The comparison of VL with VSL (when pollen is exposed to isolation solution) showed specific metabolic differences, even when both samples were harvested by vibration to avoid any possible anther tissue contamination and were lyophilised to correct for the differences in water content. Rehydration of pollen is known to have consequences on the metabolic dynamics. A study with *Capsicum annuum* pollen showed that only 30 minutes incubation in an osmotic solution at room temperature led to significant pollen rehydration and changes in sugar content (García et al. 2013). Furthermore, *Ricinus communis* seed, which, like pollen, is dry at maturity, showed marked metabolic changes upon rehydration at room temperature, with an accumulation of TCA metabolites (Ribeiro et al. 2015). In our experiments, we did not observe changes in sugar content in pollen collected and kept in the solution, but imbibed pollen accumulated a large amount of malate. Since we also observed that the detection of malate can be hindered by co-eluting salt components of the isolation solution, the increase in malate content in the rehydrated

VSL samples, as compared to the dry VL samples, might even be an underestimation. In addition, dry pollen contained more glutamate, while imbibed pollen contained more glutamine. Since glutamate is the precursor of glutamine, we speculate that pollen rehydration led to a conversion of glutamate into glutamine by a yet unknown physiological mechanism. Although we performed the isolation of pollen on ice cold solution, in order to inhibit enzymatic activities as much as possible, these results suggest that this 1 hour pollen incubation in solution, i.e. the approximate time needed to isolate (ripe or unripe) pollen from anthers, is sufficient to allow significant enzyme-mediated metabolic changes in the pollen. Support for metabolic activities even at low temperatures can also be drawn from the proteomics study on imbibing *Glycine max* seeds, in which an imbibition at 4°C for 24h showed marked changes in the protein profiles (Cheng et al. 2010). In addition to malate and glutamate/glutamine, several other compounds were altered by the one hour incubation in isolation solution, such as flavonoids, a phenylpropanoid and the amino acid serine. The use of mature dry pollen without further contact with water (i.e. isolation using the flower vibration method) thus will optimally ensure the analysis of pollen in their actual biochemical state. However, pollen at earlier stages of development cannot be released by simply vibrating the flower, as immature tomato pollen is tightly enclosed in the anther locule. Hence, cutting and squeezing anthers in an osmotic solution is unavoidable if the research aim is to collect and compare pollen of different developmental stages. Based on the above, we recommend using the terminology 'imbibed pollen' when pollen isolation is performed in any water-containing solution, to discriminate with the data from naturally dry mature pollen.

The isolation of pollen by anther squeezing causes metabolic contamination

To force the release of pollen, anthers may be cut, squeezed and vortexed. Hence, several anther tissue fractions are present in the isolation solution, such as pollen cells, locular fluid and anther walls. Even if washing steps and tissue filtration are performed, contamination of the pollen fraction by metabolites released from anther cells other than pollen cannot be excluded. We compared mature pollen isolated by vibration (VSL) and by anther squeezing (AL), which both had been incubated for the same period of time in isolation solution to prevent rehydration disparity. We observed that alkaloids and the phenolic acid feruloyl quinic acid were only present in pollen from squeezed anthers as compared with imbibed vibrated pollen. Such a difference in metabolites could reflect a contamination from anther fractions other than the pollen cells. Interestingly, some compounds, i.e. kaempferol diglucoside, feruloyl dicoumaroyl spermidine, tricoumaroyl spermidine and quercetin glucoside, were much less abundant in squeezed anthers than in imbibed vibrated pollen. This suggests that other processes than anther contamination may also affect the metabolic composition of obtained pollen using these two isolation procedures. Recently, Fragkostefanakis et al. 2016 mentioned that the collection of pollen from anthers can be sorted in two categories: the "released pollen", that is easily released by simple vortexing of the anthers in a solution, and the "unreleased pollen", which represents the pollen grains that remained on the anther wall after vortexing and need mechanical disruption (squeezing) for its release. This distinction makes us speculate that the two releasing methods applied in our study, VSL and AL, may lead to isolation of different pollen types, analogous to the released and unreleased pollen fractions mentioned by Fragkostefanakis et al.

2016. We cannot exclude that our AL samples include unreleased pollen, in addition to the released pollen. The latter may metabolically differ from the fraction of released pollen, which is likely the major fraction in the VSL samples. The difference of pollen fraction might also explain the strong difference in the presence of alkaloids. However, we previously assessed the metabolic composition of released pollen isolated with cut anthers in solution, but without the squeezing step, and observed similar alkaloid accumulation. This implies that alkaloids are more likely to come from the anther fraction than from another pollen population. Besides, several studies have also shown that metabolites can be found at the surface of the pollen, such as flavonoids and polyamines (Vantunen et al. 1991, Grienenberger et al. 2009). The filtration and washing step used to isolate AL samples could lead to a decrease of these specific metabolites. In conclusion, the pollen isolation method based on squeezing of anthers in germination medium should be addressed with care, since it could lead to anther contamination, isolation of different pollen fractions or washing away of surface compounds, in addition to the above-mentioned metabolic effect of imbibition.

Lyophilisation of pollen may prevent metabolic changes during subsequent homogenisation and extraction.

The final step of the pollen isolation procedure in solution results in a pellet of wetted pollen cells submerged in solution. There are two reasons to remove this solution and dry the pollen sample by lyophilisation: (1) pollen have different levels of water status during their development (Firon et al. 2012a). Lyophilising samples is thus required to avoid possible metabolite differences related to differential water content of developmental stages; (2) pollen contains a very high invertase activity that may lead to conversion of sugars in an aqueous environment (Pressman et al. 2002). We compared the metabolite profile of lyophilised (VSL) and non-lyophilised (VS) mature tomato pollen wetted with isolation solution as normally used for isolating pollen from different developmental stages (Pressman et al. 2012). Non-lyophilized VS samples showed a marked decrease in the disaccharide sucrose and a concomitant increase in the monosaccharides glucose and fructose, due to the higher activity of acid invertases in VS samples, most likely happening during homogenisation of the isolated pollen and/or metabolite extraction. In VS samples the mature pollen was wet and surrounded by solution, although frozen in liquid nitrogen, when pure methanol (at room temperature) was added that might thaw the tissue prior to tissue homogenization, while in VSL samples the pollen was dry before addition of methanol. We speculate that it was at this thawing step in methanol or during homogenisation when the acid invertase can be temporally activated at least in the case of the non-lyophilized pollen. Hence, we recommend to always lyophilise isolated pollen samples before metabolite extraction. As Obermeyer et al. 2013 have performed extraction of pollen metabolites of Lily (*Lilium longiflorum*) with methanol precooled at -20°C, adding a cold extraction solution can be an alternative to lyophilisation in order to prevent acid invertase activity. However, extraction at low temperatures needs further investigation, since it might also affect the extraction efficiency of less-soluble metabolites.

Tissue lyser ensures the complete homogenisation of tomato pollen

Before performing a metabolomics analysis, it is recommended to grind the tissue in order to facilitate and optimize the release of metabolites into the extraction solvent. Our results showed that manual grinding pollen using a pestle led to a relative high number of still intact pollen grains per µL, as compared to using an automatic tissue lyser with stainless steel balls. We previously observed that the presence of a high number of intact cells leads to a lower abundance of metabolites in the methanolic extracts (data not shown), suggesting that 70% methanol is not sufficient to open the intact pollen cells, due to the thick wall that surrounds the pollen. Hence, we recommend the use of a tissue lyser to grind the pollen for at least 15 minutes.

Conclusions

We have shown that different steps in the pollen isolation and extract preparation protocols usually applied in transcriptomics and proteomics research significantly influence the tomato pollen metabolome. To summarize (i) we recommend the use of a tissue lyser for grinding pollen for at least 15 minutes to ensure the optimal breaking of pollen cells, (ii) to use only pollen isolated by vibration to study the metabolic composition of mature pollen, (iii) if mature pollen is to be compared with earlier stages of development, an isolation solution is needed and the pollen should thus be qualified as imbibed, (ii) more efforts should be put on finding an isolation solution that prevents pollen rehydration. However, the suitability of any new isolation solution for metabolite extraction and metabolomics analysis needs to be assessed, (iv) the matrix effect must be verified when samples contain salts as used in standard pollen isolation solution. Alternatively, a different isolation solution should be used to prevent matrix effects (i.e. mannitol supplemented water), (v) the experimentalist needs to be aware that anther contaminations can occur during the squeezing step to release its pollen. More investigations are required to improve the purity of the pollen fraction either by increasing the washing and filtration step, or by determining the metabolic specificity of each fraction. Although we made progress to achieve a reliable metabolic profile of pollen cells, several aspects remain unanswered and deserve further investigations: How might pollen at young developmental stages react to the incubation in ice cold isolation solutions? How can we assess the purity of pollen samples? Do different fractions of pollen derived from the same anther differ metabolically? And why the conversion of sugars did not occur during pollen isolation that lasted for one hour, but apparently occurred rapidly during extraction?

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Supplementary data table 1. Annotated metabolites detected by the Orbitrap LC-MS method. The table represent the annotation information per metabolite including compound class, retention time (ret.), negative mass, Δ retention time, Δ ppm of the mass, annotation level (Ann. Level. See materials and methods section), reference (ref.) with 1, (Moco et al. 2006);2,(Iijima et al. 2008);3,(Roldan et al. 2014);4,(Ferreres et al. 2010);5, https://metlin.scripps.edu/index.php ;6,(Yang et al. 2012); 7,(Elejalde-Palmett et al. 2015) and the average per sample condition, in which AL means pollen isolated by squeezing and lyophilised; VLT: pollen lyophilised with technical replicates; VL, vibration derived pollen lyophilised; VS, vibrating pollen incubated in germination solution and non-lyophilised; VSL, vibrating pollen incubated in germination solution and lyophilised, and the fold change between the different conditions (condition 1/condition 2), if the value is positive, condition 1 has a higher value than condition 2; if the value is negative, condition 1 has a lower value than condition 2. The ‘ indicate the statistic threshold on the Tukey post-hoc test that followed the multivariate ANOVA analysis with p-value < 0.005, '" ; 0.005 <p-value< 0.01, "; and 0.01 < p-value<0.05, '. d.m., data missing. Fold changes higher than 2-fold are bold and grey highlight

Class	Annotation	Ret(min)	Mass(D)	Δ Ret.	Δ ppm	Ann. level	Ref.	Average					Fold change					
								AL	VLT	VL	VS	VSL	AL/VL	AL/VS	AL/VSL	VL/VS	VL/VSL	VS/VSL
Alkaloid	Acetoxy-tomatine	31.40	1136.5480	5.46	1.29	II	2	112211	9435	8886	8932	9834	**12.63'"**	**12.56'"**	**11.41'"**	-1.01	-1.11	-1.10
	alpha-Tomatin 1	30.07	1078.5426	7.81	1.26	I	1,2	98650	9275	22705	10067	9772	**4.34'"**	**9.80'"**	**10.09'"**	**2.26**	**2.32**	1.03
	alpha-Tomatin 2	30.58	1078.5427	7.30	1.15	I	1,2	2832536	154403	1018514	796654	686398	**2.78**	**3.56**	**4.13**	1.28	1.48	1.16
	alpha-Tomatin 3	31.17	1078.5426	6.71	1.26	I	1,2	109917	9298	58786	36409	25083	1.87	**3.02**	**4.38**	1.61	**2.34**	1.45
	Dehydrotomatine	29.43	1076.5270	0.74	1.24	III	3	517657	8779	61630	39490	21935	**8.40'"**	**13.11'"**	**23.60'"**	1.56	**2.81**	1.80
	Hydroxytomatine	23.59	1094.5375	4.85	1.28	II	2	91095	9475	8774	8485	11701	**10.38'"**	**10.74'"**	**7.79'"**	1.03	-1.33'	-1.38"
Flavonoid	Eriodictyol	27.25	287.0559	11.60	0.82	I	3	1184602	1675292	1840163	1445883	1677755	-1.55	-1.22	-1.42	1.27	1.10	-1.16
	Kaempferol	41.54	285.0402	4.15	1.05	I	1	73913	243102	312124	157331	135742	**-4.22'"**	**-2.13'"**	-1.84"	1.98'"	**2.3'"**	1.16
	Kaempferol 3,7-di-O-glucoside 1	21.50	609.1458	1.68	0.48	I	2	623444	2463791	4200595	3779680	5253875	**-6.74'"**	**-6.06'"**	**-8.43'"**	1.11	-1.25	-1.39
	Kaempferol 3,7-di-O-glucoside 2	19.88	609.1459	0.06	0.28	I	2	4017221	4102884	4740209	3955477	4797103	-1.18	1.02	-1.19	1.20	-1.01	-1.21
	Kaempferol 3-alpha-D-glucoside-7-rhamnoside	24.56	593.1505	d.m	1.00	IV	5	585494	160066	272461	114253	113851	**2.15'"**	**5.12'"**	**5.14'"**	**2.38'"**	**2.39'"**	1.00
	Kaempferol 3-O-rutinoside	27.74	593.1505	1.77	1.25	I	2,1	85059	82724	97524	87525	85924	-1.15	-1.03	-1.01	1.11	1.13	1.02
	Quercetin 3-O-glucoside	22.51	463.0878	5.11	0.93	I	2,1	9919	43956	90947	80491	178591	**-9.17**	**-8.11**	**-18.00**	1.13	-1.96	**-2.22**
	Quercetin 3-O-rutinoside-7-O-glucoside	18.17	771.1978	0.72	1.45	I	2,1,4	24344	11558	13150	12354	15868	1.85	1.97	1.53	1.06	-1.21	-1.28
	Quercetin-dihexoside	17.45	625.1404	0.03	0.93	II	2	70107	74149	76879	67344	82159	-1.10	1.04	-1.17	1.14	-1.07	-1.22
Phenolic acid	O-Feruloylquinic acid	17.63	367.1031	4.89	1.08	III	3	91287	8756	9013	9358	10706	**10.13'"**	**9.76'"**	**8.53'"**	-1.04	-1.19	-1.14
	5-Caffeoylquinic acid	14.63	353.0875	1.56	0.97	II	1,2	9732	35664	44862	9260	9536	**-4.61'"**	1.05	1.02	**4.84'"**	**4.70'"**	-1.03

Supplementary data table 1. *Cont.*

Polyamines	Caffeoyl dicoumaroyl spermidine 1	37.51	598.2553	d.m	0.94	IV	7	449187	84752	140275	127370	106902	**3.20'''**	**3.53'''**	**4.20'''**	1.10	1.31	1.19
	Caffeoyl dicoumaroyl spermidine 2	37.03	598.2554	d.m	0.84	IV	7	1163588	330334	572580	529592	493357	**2.03''**	**2.20'''**	**2.36'''**	1.08	1.16	1.07
	Caffeoyl dicoumaroyl spermidine 3	36.49	598.2554	d.m	0.84	IV	7	186891	46764	89929	118250	96659	**2.08**	1.58	1.93	-1.31	-1.07	1.22
	Caffeoyl dicoumaroyl spermidine 4	35.91	598.2554	d.m	0.84	IV	7	181710	55779	175899	240363	224369	1.03	-1.32	-1.23	-1.37	-1.28	1.07
	Dicoumaroylputrescine	32.03	379.1660	d.m	0.99	IV	5	436739	406236	402018	443945	401358	1.09	-1.02	1.09	-1.10	1.00	1.11
	Dicoumaroylspermidine	19.05	436.2237	d.m	1.11	IV	5	565759	260668	268509	307745	287374	**2.11'''**	1.84'''	1.97'''	-1.15	-1.07	1.07
	Feruloyl dicoumaroyl spermidine 1	39.85	612.2715	d.m	0.06	IV	6	323680	442561	547077	545674	601741	-1.69	-1.69	-1.86	1.00	-1.10	-1.10
	Feruloyl dicoumaroyl spermidine 2	37.78	612.2709	d.m	0.96	IV	6	8355	16082	176623	165257	209244	**-21.14'''**	**-19.78'''**	**-25.04'''**	1.07	-1.18	-1.27
	Feruloyl dicoumaroyl spermidine 3	38.71	612.2714	d.m	0.16	IV	6	203435	404607	698601	702356	781109	**-3.43'''**	**-3.45'''**	**-3.84'''**	-1.01	-1.12	-1.11
	Feruloyl dicoumaroyl spermidine 4	40.52	612.2714	d.m	0.16	IV	6	76494	244775	272942	278780	287848	**-3.57'''**	**-3.64'''**	**-3.76'''**	-1.02	-1.05	-1.03
	Feruloyl dicoumaroyl spermidine 5	39.35	612.2714	d.m	0.16	IV	6	472403	587104	733927	694232	757064	-1.55	-1.47	-1.60	1.06	-1.03	-1.09
	Feruloyl dicoumaroyl spermidine 6	40.91	612.2714	d.m	0.16	IV	6	2767894	2356448	2456268	2233258	2256545	1.13	1.24	1.23	1.10	1.09	-1.01
	Tetracoumaroylspermine 1	44.63	785.3549	d.m	0.85	IV	5	161642	120748	120342	119889	94016	1.34	1.35	1.72	1.00	1.28	1.28
	Tetracoumaroylspermine 2	43.82	785.3548	d.m	1.01	IV	5	47151	58328	68685	84102	68715	-1.46	-1.78	-1.46	-1.22	-1.00	1.22
	Tricoumaroyl spermidine 1	32.82	582.2605	d.m	0.79	IV	5	48142	67058	125230	110228	143915	**-2.6'''**	**-2.29'''**	**-2.99'''**	1.14	-1.15	-1.31
	Tricoumaroyl spermidine 2	37.28	582.2609	d.m	0.16	IV	5	240107	810591	3061910	2742367	3515201	**-12.75'''**	**-11.42'''**	**-14.64'''**	1.12	-1.15	-1.28
	Tricoumaroyl spermidine 3	38.32	582.2610	d.m	0.15	IV	5	1540230	2961134	6528551	6543318	7439896	**-4.24'''**	**-4.25'''**	**-4.83'''**	-1.00	-1.14	-1.14
	Tricoumaroyl spermidine 4	39.59	582.2612	d.m	0.47	IV	5	3793752	5148157	5804996	5810945	6000342	-1.53	-1.53	-1.58	-1.00	-1.03	-1.03
	Tricoumaroyl spermidine 5	39.04	582.2612	d.m	0.47	IV	5	5931665	8405854	9728265	9245664	9859566	-1.64	-1.56	-1.66	1.05	-1.01	-1.07
	Tricoumaroyl spermidine 6	40.13	582.2612	d.m	0.36	IV	5	6360049	9298637	9507392	8654731	9602037	-1.49	-1.36	-1.51	1.10	-1.01	-1.11

Supplementary data table 3. Annotated metabolites detected by Dionex HPLC. The table represent the average per sample condition, in which AL means pollen isolated by squeezing and lyophilised; VLT: pollen lyophilised with technical replicates; VL, vibration derived pollen lyophilised; VS, vibrating pollen incubated in germination solution and non-lyophilised; VSL, vibrating pollen incubated in germination solution and lyophilised, and the fold change between the different conditions (condition 1/condition 2), if the value is positive, condition 1 has a higher value than condition 2; if the value is negative, condition 1 has a lower value than condition 2. The ‘ indicate the statistic threshold on the Tukey post-hoc test that followed the multivariate ANOVA analysis with p-value < 0.005, ''' ; 0.005 <p-value< 0.01, ''; and 0.01 < p-value<0.05, '. d.m., data missing. Fold changes higher than 2-fold are bold and grey highlight

Compound	Average					Fold change					
	AL	VLT	VL	VS	VSL	AL/VL	AL/VS	AL/VSL	VL/VS	VL/VSL	VS/VSL
fructose	0.02	0.03	0.04	0.10	0.03	**-2.12''**	**-5.56'''**	-1.59	**-2.62'''**	1.34	**3.50'''**
glucose	0.01	0.02	0.03	0.11	0.02	**-2.81'''**	**-10.66'''**	-1.88	**-3.79'''**	1.49	**5.66'''**
Sucrose	0.29	0.38	0.42	0.20	0.41	-1.44	1.48	-1.41	**2.13'''**	1.02	**-2.09'''**

Supplementary data table 2. Annotated metabolites detected by HPLC-Q Exactive Orbitrap MS. The table represent the annotation information per metabolite including compound class, retention time (ret.), and the average per sample condition, in which AL means pollen isolated by squeezing and lyophilised; VLT: pollen lyophilised with technical replicates; VL, vibration derived pollen lyophilised; VS, vibrating pollen incubated in germination solution and non-lyophilised; VSL, vibrating pollen incubated in germination solution and lyophilised, and the fold change between the different conditions (condition 1/condition 2), if the value is positive, condition 1 has a higher value than condition 2; if the value is negative, condition 1 has a lower value than condition 2. The ‘ indicate the statistic threshold on the Tukey post-hoc test that followed the multivariate ANOVA analysis with p-value < 0.005, '" ; 0.005 <p-value< 0.01, "; and 0.01 < p-value<0.05, '. d.m., data missing. Fold changes higher than 2-fold are bold and grey highlight

Class	Compound	Ret(min)	Mass(D)	Ion Mode	Average					Fold change					
					AL	VLT	VL	VS	VSL	AL/VL	AL/VS	AL/VSL	VL/VS	VL/VSL	VS/VSL
Amino acid	Arginine	5.07	175.1189	Positive	78180	88231	118959	99520	94868	-1.52	-1.27	-1.21	1.20	1.25	1.05
	Asparagine	4.26	133.0608	Positive	10312729	14859969	16741235	15243729	14535940	-1.62	-1.48	-1.41	1.10	1.15	1.05
	GABA	5.63	104.0706	Positive	557624	850128	1128341	894005	863298	**-2.02"'**	-1.6"	-1.55'	1.26	1.31	1.04
	Glutamate	4.57	148.0604	Positive	1410844	3502390	3177529	1794343	1783774	**-2.25"'**	-1.27	-1.26	1.77"'	1.78"'	1.01
	Glutamine	4.42	147.0764	Positive	1523320	702084	508149	1215519	1026966	**3.00"'**	1.25	1.48	**-2.39"'**	**-2.02"'**	1.18
	Histidine	4.93	156.0767	Positive	167148	220256	260527	225253	206607	-1.56	-1.35	-1.24	1.16	1.26	1.09
	Isoleucine	10.9	132.1019	Positive	320327	426325	554315	452703	371493	-1.73	-1.41	-1.16	1.22	1.49	1.22
	Leucine	12.19	132.1019	Positive	333631	390490	534572	472634	382077	-1.60	-1.42	-1.15	1.13	1.40	1.24
	Lysine	4.84	147.1128	Positive	269871	301005	356883	328181	338067	-1.32	-1.22	-1.25	1.09	1.06	-1.03
	Phenylalanine	22.44	166.0862	Positive	391801	510271	636786	575912	482130	-1.63	-1.47	-1.23	1.11	1.32	1.19
	Proline	4.95	116.0706	Positive	21898084	31569637	35498807	28663155	31170401	-1.62	-1.31	-1.42	1.24	1.14	-1.09
	Serine	4.29	106.0499	Positive	251366	25413	47456	233838	221733	**5.30"'**	1.07	1.13	**-4.93"'**	**-4.67"'**	1.05
	Threonine	4.47	120.0655	Positive	360837	560408	670874	606950	490301	-1.86	-1.68	-1.36	1.11	1.37	1.24
	Valine	6.52	118.0862	Positive	769972	1055318	1307246	1111783	968484	-1.70	-1.44	-1.26	1.18	1.35	1.15
Organic acid	Malate	5.59	133.0142	Negative	157131	13470	76253	200836	241754	**2.06'**	-1.28	-1.54	**-2.63"'**	**-3.17"'**	-1.20
	Succinate	7.76	117.0193	Negative	45113	63861	79640	76425	84643	**-1.77"'**	**-1.69"'**	**-1.88"'**	1.04	-1.06	-1.11

Chapter 4

Untargeted metabolomic analysis of tomato pollen development and heat stress response

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Abstract

Developing pollen are among the plant structures most sensitive to high temperatures and a decrease of pollen viability is often associated with an alteration of metabolite content. Most of the metabolic studies of pollen have focused on a specific group of compounds, which limits the identification of physiologically important metabolites. To get a better insight in pollen development and the pollen heat stress response we used a liquid chromatography mass spectrometry platform to detect secondary metabolites in pollen of tomato (*Solanum lycopersicum L.*) at three developmental stages under control conditions and after a short heat stress at 38 ˚C. Under control conditions, the young microspores accumulated a large amount of alkaloids and polyamines whereas the mature pollen strongly accumulated flavonoids. The heat stress treatment led to accumulation of flavonoids in the microspore. The biological role of the detected metabolites is discussed. This study provides the first untargeted metabolomic analysis of developing pollen under a changing environment that can serve as reference for further studies.

Key words: Pollen development, metabolomics, untargeted analysis, heat stress, high temperature

Introduction

Sexual reproduction is a critical process in the plant life cycle and results in the production of seeds and fruits, major components of the human diet. The plant life cycle can be divided into two phases, sporophytic and gametophytic. In angiosperms, the embryo sac is the female gametophyte and is embedded within the ovule, whereas the pollen grain is the male gametophyte and is located inside the anther (Drews and Yadegari 2002; Borg et al. 2009). Pollen development is a complex process that ends with the release of mature pollen grains from the anthers at flower anthesis (Twell 2002; Honys et al. 2006; Hafidh et al. 2016). A major event during pollen development is the meiosis of the pollen mother cell, which results in formation of a tetrad of haploid microspores. Microspores are then released from the tetrads and during further microspore development, the vacuole expands and the nucleus migrates to one side of the cell. This polarization is the signal for the nucleus to undergo an asymmetrical mitotic division and produce the early bicellular pollen. The two cells of the bicellular pollen have different forms as well as different functions: the smaller, generative cell will later on give rise to the two sperm cells, whereas the surrounding, larger, vegetative cell will produce the pollen tube to ensure delivery of the sperm cells to the female gametophyte.

Tomato (*Solanum lycopersicum* L.) is an economically important crop. Pollen development of this plant is susceptible to various abiotic disturbances (Domínguez et al. 2005; Sato et al. 2000; Sato et al. 2006; Kamel et al. 2010). The development of mature and fertile pollen is one of the key processes for successful fertilization. A decrease of pollen fertility has major consequences for fruit yield (Kartikeya et al. 2012). Pollen development is particularly sensitive to high temperatures (Bokszczanin et al. 2013). A few degrees above the optimal growing temperature of tomato (18-25 ˚C) can already lead to a decrease of pollen viability. This is often associated with aberrations occurring during pollen development such as premature degeneration of the tapetum and inhibition of anther dehiscence (Suzuki et al. 2001; Matsui and Omasa 2002). In addition, the decrease in pollen viability upon heat stress is associated with a reduction of specific metabolites, such as carbohydrates and polyamines (Pressman et al. 2002; Song et al. 2002).

During its development, the young developing pollen is nurtured with metabolites coming from the tapetum and the locular fluid. Studies with specific mutants, sterile lines and biosynthetic inhibitors have shown that a decrease in the level of particular metabolites, such as the amino acid proline, glutathione, polyamines, and certain hormones is associated to a decrease of pollen fertility. These effects could be (partly) complemented through addition of the respective metabolites (Mattioli et al. 2012; Zechmann et al. 2011; Falasca et al. 2010; Cheng et al. 2006; Goto and Pharis 1999; Ishiguro et al. 2001). Besides their role in pollen nutrition or signaling, metabolites can serve as protectants against environmental stresses. For instance, flavonoids and polyamines can act as scavengers of reactive oxygen species (ROS) (Rice-Evans et al. 1996; Ha et al. 1998). Despite these examples, the current knowledge on the role and importance of primary and secondary metabolites during pollen development is still limited (reviewed by Paupière et al. 2014).

Most studies addressing metabolic changes during pollen development have focused on the detection of a restricted group of target compounds. Over the last decade, the use of mass spectrometry-based metabolomics approaches, e.g. gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS), made it possible to detect simultaneously hundreds to thousands of metabolites in a single extract. This has provided a more comprehensive insight in various aspects of plant development and stress responses

(Kaplan et al. 2004; Kim et al. 2007; Osorio et al. 2011), including the dynamics of the primary metabolome during pollen germination in lily (*Lilium longiflorum*) (Obermeyer et al. 2013).

Our current understanding of the physiological processes occurring during tomato pollen development under optimal conditions and in response to heat stress is largely based on proteomics and transcriptomics data (Chaturvedi et al. 2013; Honys and Twell 2004). Metabolomics approaches are necessary to complement the omics-derived knowledge and enable developing models for the system as a whole. The objective of this study was to explore the composition and dynamics of the secondary metabolome of tomato pollen, under a normal temperature of 22 ˚C and after a short heat stress of two hours at 38 ˚C. Our results show that the most significant metabolic changes involved the conjugation and relative abundance of flavonoids, polyamines and alkaloids.

Materials and Methods

Plant materials and growing conditions

Tomato (*Solanum lycopersicum* L.) seeds, cultivar Micro-Tom, were obtained from the National Bioresource Project in Japan (TOMJPF00001). Plants were grown in a climate chamber (MC1600 Snijders Labs, The Netherlands) under constant temperature of 22 ˚C, with 12/12 hour photoperiod and a relative humidity of 60%. Light was provided by LED lamps (Philips Green Power LED DR/B/FR 120, ≈ 250 μmol/m^2s).

When approximatively five to eight flowers had appeared on the plants, they were subjected to a heat stress of 38 ˚C or kept at control conditions. After 2 hours of treatment pollen were harvested as described below. Treatments were performed in a staggered fashion, with 30-minute gaps between plants to reduce the time that samples were kept on ice during pollen isolation and were done over a six-day period such that three biological repetitions were collected for each of the heat stress and the control conditions, each consisting of a pool of pollen derived from flower buds of 10 plants.

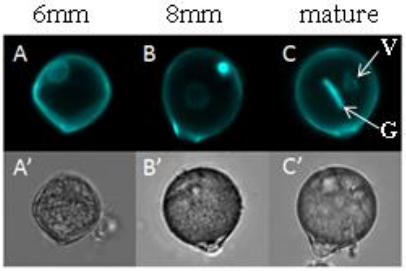

Figure 2. Pollen development of S. lycopersicum Micro-Tom cv. Pollen of polarized microspore stage is represented in Fig. A-A'; Pollen of early bicellular stage is represented in Fig. B-B'; Pollen of mature pollen stage with a vegetative nucleus (V) and a generative nucleus (G) is represented in Fig. C-C'. Sizes of the anther are indicated above the pictures. A-C, fluorescence microscopy after DAPI staining to visualize the nucleus; A'-C', light microscopy

Determination of pollen developmental stages

To determine pollen developmental stages, flower buds of Micro-Tom were measured, and anthers were cut into 2-3 pieces and subsequently placed in a 0.3 M mannitol solution. Pollen were released from the anther by vortexing, precipitated by centrifugation at 1000 rpm and incubated with 70% ethanol at room temperature for 30 minutes. The supernatant was removed

after one minute of centrifugation, and pollen were incubated with 10-30 µl of DAPI 2-5 µg/ml in the dark for one hour. One droplet of DAPI-stained pollen suspension was transferred to a glass slide and analysed with a Leica TCS SP2 AOBS Confocal Laser Scanning Microscope. Three pollen developmental stages were used for this study: polarized microspore, early bicellular pollen and mature pollen (Figure. 1).

Pollen harvesting

The pollen harvesting procedure was adapted from the protocol of Firon et al. 2006. Flower buds were removed from the plants and kept on ice. The bud size was determined with a ruler from the base of the anther until the tip including the sepals. Petals, pistil and sepals were removed with forceps before the anther cone was cut into pieces. Anther pieces were transferred into a 1.5-ml Eppendorf tube containing 500 µL ice cold germination solution and stored on ice. The germination solution consisted of 1 mM KNO_3, 3 mM $Ca(NO_3)_2.4H_2O$, 0.8 mM $MgSO_4.7H_2O$ and 1.6 mM H_3BO_3 dissolved in distilled water. Anthers were squeezed with a 1 ml pipette tip to release the pollen. After vortexing, the solution was filtered through four layers of miracloth (Calbiochem) and then centrifuged at 300 g for two minutes at 4 ˚C followed by a short spin at maximum speed of 17000g. Supernatant was removed and the pollen pellet was washed with 100 µL of ice cold germination solution, followed by centrifugation. This was repeated once and then the pollen pellet was transferred into a pre-weighted 2-ml Eppendorf tube, frozen in liquid nitrogen, stored at -80 ˚C and then freeze dried. Pre-weighted 2-ml Eppendorf tubes containing the freeze dried pollen were subsequently weighted to determine the weight of the pollen.

Pollen viability

Pollen quality was analysed by *in-vitro* pollen germination and pollen viability tests. Flower buds of 6 and 8 mm were treated as described above, labelled and analysed upon anthesis. Open flowers were analysed directly after treatment. Five plants were used per treatment and stage; per plant one to four open flowers were analysed. Petals, pistil and sepals were removed and the anther cone was cut into slices and incubated in a humid atmosphere at room temperature for 30 minutes to allow slow hydration of dry pollen. Anther pieces were transferred to an Eppendorf tube containing germination solution, as described above, supplemented with 5% sucrose and 25% polyethylene glycol 4000. The sample was vortexed for 10 seconds to release pollen and incubated for two hours at room temperature while rotating slowly. Per open flower at least 100 pollen grains were counted and scored as germinated, viable or dead. Pollen were considered as germinated when the pollen tube length exceeded the pollen diameter, as viable when pollen were hydrated and as dead when pollen grains did not hydrate. A plant was taken as a biological replicate, leading to at least five biological replicates per developmental stage and per treatment.

Metabolite extraction

Semi-polar metabolites extractions were carried out at room temperature using water:methanol:chloroform separation, as previously described by Wahyuni et al. 2013. 300 µL of 70% methanol was added to each pollen extract as well as three 2-mm stainless steel beads. Samples were homogenized for 15 minutes using a Tissuelyser (Qiagen®) followed by sonication for 10 minutes and centrifugation for 10 minimum at maximum speed of 17000g. 200 µL of the supernatant was transferred into a new 1.5 ml Eppendorf tube containing 200 µL of 70% methanol and filtered with a 0.2 µm polytetrafluoroethylene filter. The weight of original freeze dried pollen samples varied from 1.96 to 4.8 mg. To avoid a situation where the sample weight-related quantitative metabolic differences would go beyond the linear detection

range of the mass spectrometer detector, each extract was diluted with 70% methanol proportionally to the difference between the weight of its original freeze dried sample and the sample with the minimal weight. The final extract was transferred into a 2 ml crimp glass vial with insert.

Metabolic profiling

Semi-polar metabolites were separated using a C-18 reversed phase liquid chromatography column and detected by quadrupole time of flight mass spectrometry (LC-QTOF-MS) with negative electrospray ionization. The LC-MS was also coupled to a photodiode array detector allowing spectrophotometric detection. 10 µL of extract were injected and separated using a binary gradient of water (A) and acetonitrile (B), both acidified with 0.1% formic acid, with a flow rate of 0.19 ml/min. The initial solvent composition consisted of 95% of A and 5% of B; increased linearly to 35% A and 65% B in 45 min and maintained for 2 min. The column was washed with 25% A and 75% B for 5 min and equilibrated to 95% A and 5% B for 2 min before the next injection as previously described by De Vos et al. 2007 and Wahyuni et al. 2013. The data were recorded with MassLynx software.

Metabolite data processing

LC-QTOF-MS data were processed using MetAlign software (available from www.metalign.nl) to correct for the baseline and noise and to perform a mass spectral alignment of chromatograms as previously described by (Tikunov et al. 2005 ; De Vos et al. 2007). MetAlign output was reduced by omitting mass data showing values lower than the detection threshold (20 ion counts) in more than two samples once the estimated peak signal was subtracted to the estimated noise signal. Compound mass spectra and quantitative ions were extracted from the modified MetAlign outputs using a method described in (Tikunov et al. 2012) by MsClust software (available from www.metalign.nl). MSClust output was reduced by keeping compounds that were quantitatively present in all the replicates of one of the experimental treatments: heat stress or control. LC-QTOF-MS masses were kept for analysis when at least one sample had a relative abundance higher than 200 counts. If a quantitative ion automatically selected by MSClust showed saturation of the MS detector, this ion was replaced by its second or third isotopic ion. MSClust output files were then used for compound annotation. Putative annotation of ions was performed with an in-house metabolite database and metabolite online databases Dictionary of Natural Products (http://dnp.chemnetbase.com/) and METLIN (http://metlin.scripps.edu/). The annotation of compounds was performed according to the Metabolomics Standards Initiative requirements (Sumner et al. 2007). Identified compounds were annotated level I when NMR was performed on annotated compounds from the in-house library, level II when an analytical standard was used to annotate the compounds from the in-house library, or when a tandem mass spectrometry was performed and level III when compounds were annotated based on their mass. Annotation level of compounds are indicated in the supplementary data Table 1.

Tandem mass spectrometry

An MS^3 analysis was performed using Acquity UPLC – PDA e Detector (Waters) coupled to LTQ Orbitrap XL mass spectrometer (Thermo Scientific). The MS^3 analysis was performed as previously described by van der Hooft et al. 2011 and Wahyuni et al. 2011. Negative masses 598.25, 612.27, 582.26 and 785.35 have been submitted to MS/MS and MS^3 using the most intense ion within a 3 Da window around the selected masses, a CID activation type and a normalized collision energy of 35.0. Fragmentation outputs were analysed in Excalibur to establish a fragmentation tree of each mass and allow the identification.

Determination of flavonoids and polyamines total abundance

The UV spectrum was obtained from the photo diode array detector of the LC-QTOF-MS. Peak areas of flavonoids were obtained by integration at 340 ± 15 nm. Peak areas of conjugated polyamines were obtained by integration at 260 ± 15 nm.

Statistical analysis

All the statistical analyses were performed with Genstat 18th Edition except mentioned otherwise. For the metabolomics analysis three biological replicates per developmental stage and treatment were used. Statistical analyses were performed on log2 transformed values. A univariate ANOVA analysis was performed for each annotated metabolite with a treatment structure: condition factor x development factor and a block structure considering the number of observations within plants. This block structure was introduced in order to overcome the dependency between stages, since the samples at the three developmental stages were taken from the same pool of plants. Due to the large number of variables generated by metabolomics analysis, 41 secondary metabolites, the statistically significant p-value threshold was adjusted for multiple testing. A P-value of 0.01 was used as a threshold. The ANOVA of metabolites with a statistically significant p-value was followed by a Bonferroni's post hoc test to correct for the number of analysed pair-wise comparisons; p-values lower than 0.05 were considered statistically significant. A principal component analysis was performed on log2 transformed and mean centered values for both metabolomics platforms with GeneMaths XT v.2.12. For the short heat stress pollen viability test, at least five biological replicates per developmental stage and per treatment were used. The IBM SPSS statistic software package 20 (www.ibm.com) was used to perform the ANOVAs on the ratio of viable and germinated pollen followed by a Tukey's post hoc test; p-values lower than 0.05 were considered statistically significant.

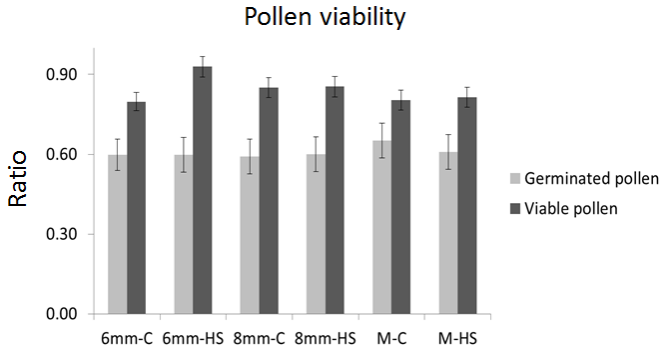

Figure 2. Pollen viability under control and heat stress treatments. 6mm, polarized microspore; 8mm, early bicellular pollen; M, mature pollen; C, control condition; HS, heat stress treatment. No statistically significant differences were found between control and heat stress treatments for each of the developmental stages (Tukey test, p-value <0.05). Bars represent the standard error of the mean.

Results

Pollen viability

In order to determine the impact of a short heat stress on tomato pollen quality, plants were kept in control temperature or exposed to 38 ˚C for two hours. Buds of different developmental

stages were labeled and analysed upon anthesis: 6 mm buds, i.e. containing mostly polarized microspores, 8 mm buds, containing early bicellular pollen and open flowers, containing mature pollen (Figure. 1). The heat treatment did not lead to statistically significant changes in the proportion of germinated or viable pollen in the three pollen developmental stages tested (Figure 2, p-value >0.05). This means that potential differences in metabolite abundance are likely to reflect the cellular heat stress response, rather than deterioration of (part of) the pollen grains.

Table 1 Identification of hydroxycinnamic acid conjugated polyamines by $MS^{2,3}$ identification. Sperm, spermidine; coum, coumaroyl; caff, caffeoyl; fer, feruloyl; spn, spermine.

Compound	$[M-H]^-$	MS2	MS3	Name	Formula
Compound 1	598	358, 436 [sperm + coum + coum -H]$^-$, 462, 478, 452 [sperm+coum+caff-H]$^-$	358→315,358→300, 452→332 [sperm + caff + CO-H]$^-$, 452→316, 462→342, 478→342. 478→358	Caffeoyl dicoumaroyl spermidine	$C_{34}H_{37}N_3O_7$
Compound 2	612	492, 372, 476, 466 [sperm + fer + coum -H]$^-$, 462 [sperm + coum + coum+CO -H]$^-$	492→299, 492→161, 492→372 , 492→316 [sperm+coum+CO-H]$^-$, 492→342, 492→175 [feruloyl-2H]$^-$, 492→145 [sperm]$^-$, 372→175 [feruloyl-2H]$^-$, 476→145, 466→320, 462→145 , 462→342	Feruloyl dicoumaroyl spermidine	$C_{35}H_{39}N_3O_7$
Compound 3	785	545, 639 [spn+coum+coum+coum-H]-, 665 [spn +coum+coum+coum+CO-H]$^-$,	545→399, 665→545 [spn+coum+coum+2CO-H]$^-$	Tetracoumaroyl spermine	$C_{46}H_{50}N_4O_8$
Compound 4	582	462 [sperm+coum+coum+CO-H]$^-$, 436 [sperm+coum+coum-H]$^-$, 342	462→342 ' 462→316 [sperm+coum+CO-H]$^-$, 462→299, 436→316[sperm+coum+CO-H]$^-$, 436→273, 342→299,342→256	Tricoumaroyl spermidine	$C_{34}H_{37}N_3O_6$

To analyse changes in secondary metabolites during pollen development and upon high temperature stress a LC-QTOF-MS analysis was performed on semi-polar extracts of tomato pollen. In total 41 putative compounds were detected in different pollen samples of which 38 could be annotated. Most of the putatively identified secondary metabolites belonged to three major groups: flavonoids, polyamines and alkaloids (Supplementary data Table 1). Polyamines showed a large structural diversity and their peaks were the most intense in the chromatograms (Supplementary data Figure. 1). To shed light on the structural variation of the polyamines in tomato pollen, the major parent ions of 598.25 Da, 612.27 Da, 785.35 Da and 582.26 Da, representing the most abundant unknown polyamines, were subjected to MS^3 fragmentation (Table 1). We found that spermidine was conjugated with coumaroyl (coum), caffeoyl (caff) and feruloyl (fer) moieties, while spermine was conjugated with coumaroyl moieties only. The fragmentation of the mass 598 gave three relevant fragments: 436 [sperm+coum+coum-H]$^-$, 452 [sperm+coum+caff-H]$^-$ and 332 [sperm+caff+CO-H]$^-$, which led to the identification of caffeoyl dicoumaroyl spermidine, $C_{34}H_{37}N_3O_7$. The fragmentation of the mass 612 gave four relevant fragments: 466 [sperm+fer+coum-H]$^-$, 462 [sperm+coum+coum+CO-H]$^-$, 316 [sperm+coum+coum+CO-H]$^-$, 175 [feruloyl-2H]$^-$ and 145 [sperm]$^-$, which led to the identification of feruloyl dicoumaroyl spermidine, $C_{35}H_{39}N_3O_7$. The fragmentation of the mass 785 gave three relevant fragments: 639 [spn+coum+coum+coum-H]$^-$, 665 [spn+coum+coum+coum+CO-H]$^-$ and 545 [spn+coum+coum+2CO-H]$^-$, which led to the identification of tetracoumaroyl spermine. The fragmentation of the mass 582 gave three relevant fragments: 462 [sperm+coum+coum+CO-H]$^-$, 436 [sperm+coum+coum-H]$^-$ and 316[sperm+coum+CO-H]$^-$, which led to the identification of tricoumaroyl spermidine.

Developmental changes in secondary metabolism of Micro-Tom pollen

Principal component analysis (PCA) of the LC-QTOF-MS data revealed three groups corresponding to the three pollen developmental stages (Figure 3A). The first principal component represented most of the differences among the samples explaining 74.8% of the variance. This was due to the difference between the earliest of the three stages, the polarized microspores, and the two later developmental stages. The variance of the first component was

mainly due to two compounds, the flavonol kaempferol dihexoside and the alkaloid beta-tomatine, which showed a contrasting accumulation pattern during pollen development.

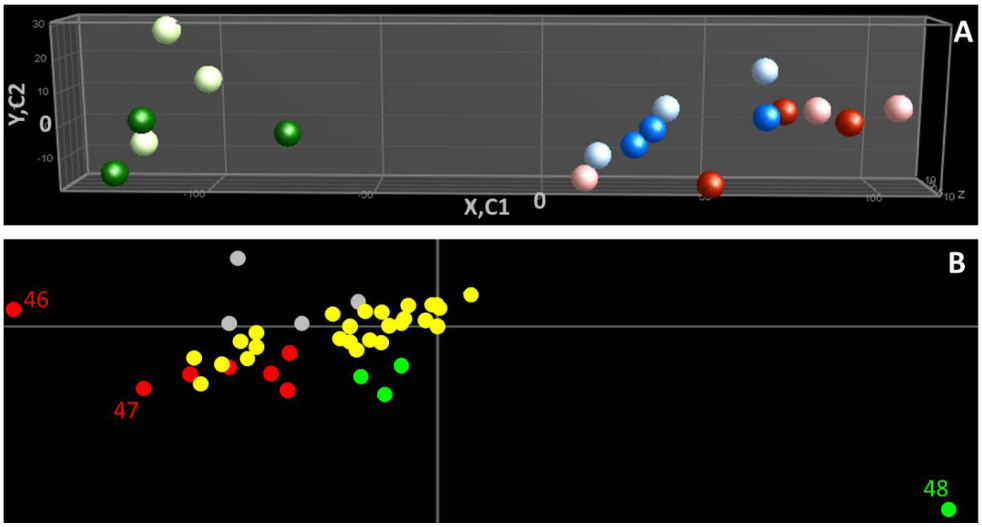

Figure 3. Principal component analysis (PCA) of secondary metabolism. The PCA of the samples is represented in plot A with light green dots for 6mm control (C) sample, dark green dots for 6mm heat stress (HS) sample, light blue dots for 8mm-C, dark blue dots for 8mm-HS, light pink dots for M-C, and red dots for M-HS. Component 1 (C1), component 2 (C2) and component 3 (C3) explain 74.8%, 10.3% and 4.3% of the observed variation, respectively. The metabolites responsible for the variation among the samples are represented in plot B with grey dots for unknown compounds, red dots for alkaloids, yellow dots for conjugated polyamines, and green dots for flavonoids. 46, beta-tomatin; 47, tomatin and 48 kaempferol dihexose. The PCA was performed on log2 transformed and mean centred values.

Sixteen annotated metabolites showed significant differences between the developmental stages under control conditions (p-value <0.005, Supplementary data Table 1). During pollen development the alkaloids alpha and beta-tomatin significantly decreased between polarized microspore and mature pollen stage by 2.3-fold and 16.6-fold, respectively (Figure 4A). Kaempferol dihexoside significantly increased by 16.7-fold in mature pollen compared to polarized microspores (Figure 4B). Nineteen (out of 25) different spermidine conjugates significantly decreased during pollen development: four isomers of dicoumaroyl spermidine and two isomers of feruloyl coumaroyl spermidine significantly decreased between polarized and bicellular stage (Figure 4C and D). Different isomers of other conjugated polyamines such as caffeoyl dicoumaroyl spermidine, tricoumaroyl spermidine, diferuloyl coumaroyl spermidine and feruloyl dicoumaroyl spermidine also showed a significant decrease during pollen development (Supplementary data Table 1). In general, the abundance of all the alkaloids and most of the polyamines showed a tendency to decrease during pollen development, although the majority of those differences were not significant (Supplementary data Table 1).

Differences in ionization efficiency make a quantitative comparison of different flavonoids and spermidines impossible. However, the use of a photodiode array (PDA) detector allowed us to compare the relative abundance of the individual and the total abundance of all flavonoids and polyamines within each sample, by measuring their absorbance at 340 ± 15 nm and 260 ± 15 nm, respectively (Figure 5, Supplementary data table 2). During pollen development, the total abundance of flavonoids increased significantly by 8.5-fold from polarized microspore to mature pollen stage (Figure 5A). Kaempferol dihexoside was the most abundant flavonoid form

among the detected flavonoids in mature pollen and significantly increased from polarized microspores to mature pollen. The total abundance of conjugated polyamines decreased with 37% from polarized microspore to early bicellular pollen stage (Figure 5B). At the level of individual compounds, the total abundance of dicoumaroyl spermidine, diferuloyl coumaroyl spermidine, feruloyl coumaroyl spermidine, feruloyl dicoumaroyl spermidine and tricoumaroyl spermidine forms significantly decreased between polarized and early bicellular pollen stage while the total abundance of caffeoyl dicoumaroyl spermidine forms significantly decreased from early bicellular to mature pollen stages (Figure 5B).

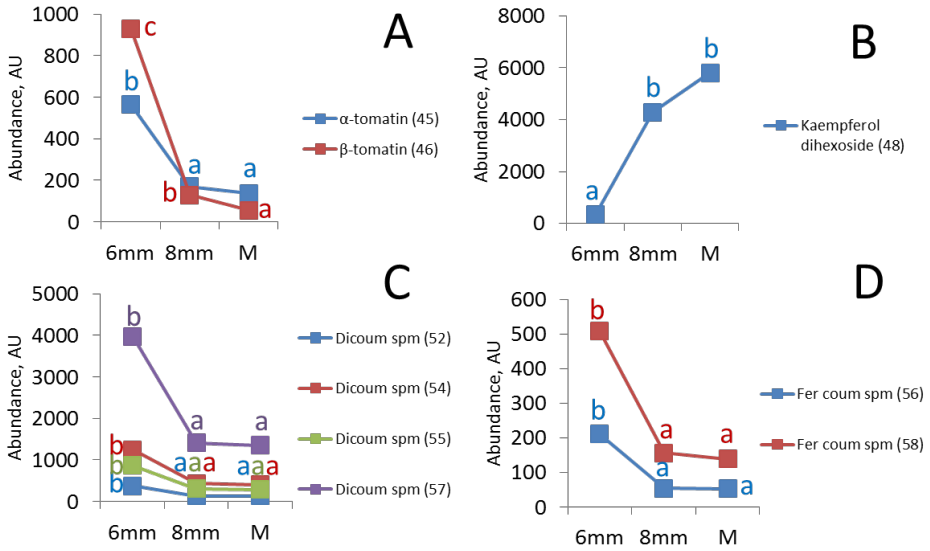

Figure 4. Secondary metabolite profiles during pollen development under control condition. The values per stage represent the average value per stage of both control and heat conditions. Only metabolites showing statistically significant differences between the developmental stages are represented. Abundances of alkaloids are represented in panel A, flavonoids in panel B, dicoumaroyl spermidine isomers in panel C and caffeoyl dicoumaroyl spermidine isomers in panel D. 6mm, polarized microspore; 8mm, early bicellular pollen; M, mature pollen; C, control condition; Dicoum, dicoumaroyl; Sperm, spermidine; Fer, feruloyl. Letters show statistically significant differences between the developmental stages per metabolite. Similar letters per metabolite indicate that there was no significant difference between the stages. Differences were considered statistically significant when the p-value of the ANOVA test was lower than 0.01 and the p-value of the Bonferroni post hoc test was lower than 0.05.

The effect of a short heat stress on pollen secondary metabolism

The PCA of the secondary metabolites did not show a clear separation of the two temperature treatments, neither in the first, nor in the second or third principle component (Figure 3A). In line with the PCA, two-way ANOVA revealed that among the 38 putatively annotated compounds, none showed significant differences between control and heat stress (Supplementary data Table 1). However, the total level of flavonoids was significantly, 2-fold, higher after the short heat stress compared to control conditions in polarized microspores (Figure 6, Supplementary data Table 3). Although the unidentified flavonoid 1 showed a significant two-way interaction (Supplementary data Table 2), it did not meet the criteria of the Bonferroni post hoc test (Supplementary data Table 3) The individual flavonoids all showed the same trend, but did not reach our statistical threshold. Neither polyamine levels nor alkaloid levels seemed to be affected by the heat stress applied.

Discussion

The objective of this study was to obtain a broad overview of the changes in the secondary metabolome during tomato pollen development under control conditions and after a heat stress treatment, by using a non-targeted metabolomics approach. Data on secondary metabolites in developing pollen are still limited and only a few targeted approaches have been used in the past (Paupière et al. 2014).

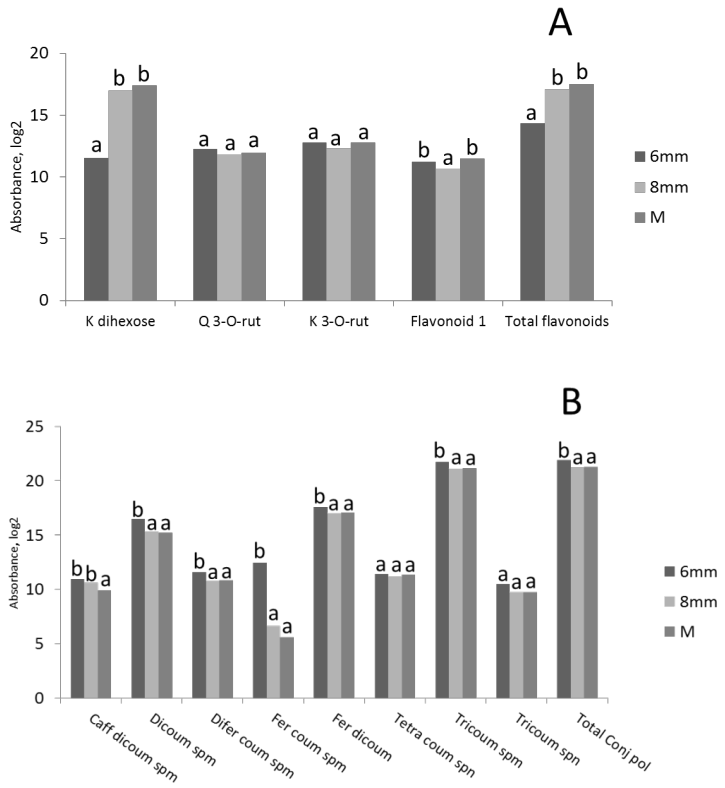

Figure 5. Absorbance profiles of secondary metabolites during pollen development under control condition. The values per stage represent the average value per stage of both control and heat conditions. The absorbance of flavonoids detected using photodiode array (PDA) at 340 ± 15 nm are represented in panel A. The abundance of polyamines detected by PDA at 260 ± 15 nm are represented in panel B. Isomers of each conjugated polyamine were summed up to represent the total abundance of each conjugated form. 6mm, polarized microspore; 8mm, early bicellular pollen; M, mature pollen; C, control condition. K, kaempferol; Q, quercetin; rut, rutinoside; coum, coumaroyl; caff, caffeoyl; fer, feruloyl; spm, spermidine; spn, spermine; total conj. Pol, total conjugated polyamines. Letters show statistically significant differences between the developmental stages per metabolite. Similar letters per metabolite indicate that there was no significant difference between the stages. Differences were considered statistically significant when the p-value of the ANOVA test was lower than 0.01 and the p-value of the Bonferroni post hoc test was lower than 0.05.

Metabolomics analyses were performed on three pollen developmental stages: polarized microspores, early bicellular pollen and mature pollen. It is worth mentioning that young, developing pollen such as at the polarized microspore and early bicellular pollen stages, are tightly enclosed in the anthers and cannot be easily released from them. As was done in previous studies, to achieve the release of early stage pollen, they were collected in an osmotic

germination solution (Firon et al. 2006; Pressman et al. 2002; Chaturvedi et al. 2013). However, the incubation of mature dry pollen in a solution during pollen isolation leads to pollen rehydration and therefore the studied mature pollen needs to be considered as imbibed pollen. Also, despite the precautions taken during isolation, we cannot exclude that the squeezing of anthers in the germination solution to release young microspores may lead to some contamination from the anther tissue and/or the locular fluid.

Pollen development

Polyamines

Polyamines are known to be widely present in the plant kingdom. They can be found in a free form, bound to proteins or conjugated with other metabolites such as phenolic acids (Aloisi et al. 2016). The majority of the polyamine forms detected in this study of developing pollen were spermidine acylated with hydroxycinnamic

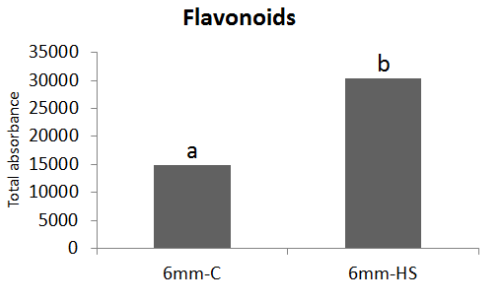

Figure 6. Total absorbance of flavonoids under control and heat stress treatments. The total abundance of flavonoids was determined by the sum of the photodiode array absorbance of individual flavonoids at 340 nm ± 15. Stars underline statistically significant differences between 6mm-C and 6mm-HS. Letters show statistically significant differences between the developmental stage per metabolite. Similar letters per metabolite indicate that there was no significant difference between the stages. Differences were considered statistically significant when the p-value of the ANOVA test was lower than 0.01 and the p-value of the Bonferroni post hoc test was lower than 0.05.

moieties such as coumaroyl, caffeoyl and feruloyl groups, commonly known as hydroxycinnamic acid amides. These compounds have also been found in *Arabidopsis thaliana* mature pollen (Handrick et al. 2010). The total level of conjugated polyamines was 37% lower in late pollen developmental stages compared with polarized microspores. The conjugated forms of polyamines are considered to be relatively inactive compared to free forms (Bagni et al. 1994), although the physiological role of acylated polyamines has not been clarified completely yet. It has been suggested, for example, that acylation may promote the stability and compartmentation of polyamines (Bassard et al. 2010), which is of importance for cell types, such as pollen, in which preservation of resources plays a crucial role. Recently, it was shown that a mutation of a spermidine hydroxycinnamoyltransferase in *Arabidopsis thaliana* resulted in a lower level of conjugated spermidine together with pollen wall irregularities (Grienenberger et al. 2009) and defects in seed set (Fellenberg et al. 2008). Our limited understanding of the role of conjugated polyamines in plant development and stress response might, at least in part, be due to the fact that most of the genes involved in their conjugation have not been identified yet (Tiburcio et al. 2014). Nevertheless, several roles of polyamines have been demonstrated. With two to four nitrogen atoms, polyamines may play a role in the nitrogen metabolism, as was already suggested by Altman and Levin 1993. Additionally,

polyamines act in the protection against environmental stresses, since they scavenge reactive oxygen species and preserve the integrity of membranes (Ha et al. 1998; Das and Misra 2004). Polyamines are also known to be important compounds for pollen development. For instance, inhibition of the polyamine pathway by pharmacological or genetic means led to a reduction of pollen viability in kiwi (*Actinidia deliciosa*), rice *(Oryza sativa)* and tomato (Falasca et al. 2010; Chen et al. 2014; Song et al. 2001). Finally, polyamines play a role in pollen tube growth through the organization of the cytoskeleton and the cell wall deposition of the pollen tube (Aloisi et al. 2016). A proper cytoskeleton organization is required to ensure the cell expansion and transport of the two sperm cells. Incorporation of polyamines in actin filaments directly affects actin polymerization and subsequent pollen tube growth. In addition, proteins conjugated with polyamines were found in the cell wall of the growing pollen tube, which is another indication that polyamines are involved in pollen tube growth (Di Sandro et al. 2010). It is currently unclear if the observed changes in the relative abundance of specific polyamine-conjugates during pollen development and the small, but significant (37%) decrease in the total level of conjugated polyamines have functional consequences in relation to pollen development and the subsequent fertilization processes, such as pollen tube growth.

Flavonoids

We observed a strong increase in the total abundance of flavonoids during tomato pollen development. Kaempferol, conjugated with two yet unknown hexose sugar moieties, was found to be the predominant compound of this class. In line with our study, kaempferol glycosides were the most abundant flavonoid forms in pollen of petunia (*Petunia hybrida L.*) (Zerback et al. 1989). Many studies have shown the importance of flavonoids for pollen viability, especially through the characterization of *CHALCONE SYNTHASE* (*CHS)* mutants, which show decreased pollen germination in many species, including petunia (Taylor and Jorgensen 1992), maize (*Zea mays*) (Coe et al. 1981) and tomato (Schijlen et al. 2007). The strong accumulation of flavonoids observed in imbibed mature pollen suggests an important role for these compounds in pollen development, pollen germination or pollen tube growth. Although the mechanisms of flavonoid action are still unclear, it has been suggested that flavonoids contribute to pollen wall plasticity to allow for fast pollen tube growth (Derksen et al. 1999). Flavonoids are also powerful antioxidants that protect against environmental stresses by scavenging reactive oxygen species (Rice-Evans et al. 1996). It is important to note that only conjugated forms of flavonoids were detected. These conjugated forms are considered as the storage form of flavonoids. In the petunia *CHS* mutant, pollen germination could only be rescued by adding flavonol aglycones to the *in-vitro* germination medium, while flavonol glycosides were not effective (Mo et al. 1992). It has been hypothesized that flavonol glycosides act as a reserve to provide the aglycone form when needed through the action of a glycosidase. In line with this, we assume that the conjugated flavonoids accumulated in mature pollen will be converted into aglycone forms and used during pollen germination and pollen tube growth.

Alkaloids

The two glycoalkaloids α and β-tomatin were profoundly accumulated in young polarized microspores compared to mature stages. Glycoalkaloids are well known for their ability to protect against biotic stresses, they inhibit the growth of fungi and are toxic for insects (Friedman 2002). The knowledge on the role of glycoalkaloids in pollen is scarce, but the different levels of glycoalkaloids observed in different stages of pollen development might be important to ensure an optimal defense against biotic attacks during plant reproduction. This

idea would agree with the finding that the concentration of α-tomatin has been reported to be 2-fold higher in flowers than in leaves of tomato plants (Kozukue et al. 2004).

In addition to the annotated compounds discussed above, three unknown compounds showed statistically significant differences between the different developmental stages. Elucidating their identities might be relevant to increase the knowledge of the metabolic dynamics occurring during pollen development.

Impact of heat stress

It is well known that a rise of temperature leads to a decrease of pollen viability (Muller and Rieu 2016). In tomato for instance, both a short heat shock and a long-term mildly elevated growth temperature can lead to a significant reduction of pollen numbers and germination potential (Firon et al. 2006; Dane et al. 1991; Fragkostefanakis et al. 2016). To be able to study the effect of heat treatment on pollen metabolome, we applied a heat treatment that did not affect the viability of resulting mature pollen. Indeed, a more severe heat shock would be required to affect pollen development (Iwahori 1965). However, short-term exposure to non-damaging high temperatures is known to lead to acquired thermo-tolerance, i.e. improved ability of pollen to withstand subsequent damaging temperatures (Firon et al. 2012; data not shown). Thus, it might be expected that the treatment applied here elicits adaptive metabolic responses in pollen. We did not observe a strong impact of our heat treatment on the pollen metabolome after two hours of heat stress; an increase in the total abundance of flavonoids in polarized microspore stage was the only significant metabolic alteration detected. Flavonoids play an important role in the detoxification of ROS (Rice-Evans et al. 1996). Under temperature stress, ROS often accumulate and play a role in signaling (Driedonks et al. 2015). However, at the same time their accumulation is harmful for the cell, explaining why temperature stress is often associated with accumulation of ROS scavengers (Suzuki and Mittler 2006). We therefore suggest that the accumulation of flavonoids upon heat is involved in the protection against ROS.

The weak metabolic response in the three pollen developmental stages contrast with the findings of Kaplan et al. 2004, who showed that a short heat stress of 40 ˚C led to a dynamic response of the primary metabolome in leaves of Arabidopsis. Given that we analyzed the pollen directly after heat application, it is likely that the increase in flavonoids is part of the very initial metabolic response to heat stress and that further responses occur at later time points. The use of earlier developmental stages such as meiotic microspore, known to be most sensitive to short heat stress, could potentially offer a stronger metabolic response. However, the low metabolite content of this stage makes the use of metabolomics analysis challenging (data not shown). As summarized by Mesihovic et al. 2016 the determination of a heat stress regime is a critical aspect when studying heat stress responses and this strongly influences the final outcome. This study was a first attempt to study the influence of heat stress on the secondary metabolome in pollen. In future studies, we aim to determine the metabolic response of pollen to heat stress at several time points after a given heat treatment and at different developmental stages. This should lead to a more comprehensive picture of the dynamics in the secondary metabolite response of tomato pollen to heat stress.

To summarize, this study was the first attempt to unravel secondary metabolites changes from microspore to mature pollen stage under changing environment and can serve as reference for future investigation of these processes. We provided an untargeted analysis of secondary metabolites in developing tomato pollen grains. Young pollen stages accumulated specific conjugated polyamines and alkaloids whereas mature pollen stage accumulated more flavonoids. The short heat stress of 2 hours at 38 ˚C led to an increase in total content of

flavonoids upon stress in the microspore stage. The accumulation of flavonoids may protect against oxidative damage induced by the temperature increase.

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Supplementary data Table 1. Abundance of metabolites detected by the LC-QTOF-MS. Metabolites are organized by classes (alkaloid, flavonoid and polyamine). The average abundance was calculated from the biological replicates per condition (from 6mm-C to M-HS), and the average per stage was calculated (from 6mm to M). Letters show statistically significant differences between the developmental stages per metabolite. Similar letters per metabolite indicate that there was no significant difference between the stages. Differences were considered statistically significant when the p-value of the ANOVA test was lower than 0.01 and the p-value of the Bonferroni post hoc test was lower than 0.05. Boxes represent p-values of the ANOVA test lower than 0.01. N°, compound number; Rt, retention time; An, annotation; ref, reference; 6mm, polarized microspore; 8mm, early bicellular pollen; M, mature; C, control condition; HS, short heat stress condition; Cor, corrected; Stag, developmental stages; Con, condition; Int, interaction stages*conditions. 1,(Iijima et al. 2008); 2,(Moco et al. 2006); 3, (Tikunov et al. 2010); 4,(Roldan et al. 2014); 5, metlin.com; 6, Table 1; 7 (Handrick et al. 2010)

									Average of abundance									Fold change			ANOVA p-value		
Class	Compound	N°	Rt (min)	Negative mass (Da)	An. level	Ref.	Δ Rt	Δ ppm	6mm-C	6mm-HS	8mm-C	8mm-HS	M-C	M-HS	6mm	8mm	M	6mm vs 8mm	6mm vs M	8mm vs M	Stag.	Con.	Int.
Alkaloid	Hydroxytomatine	41	26.39	1048.5374	II	1	0.28	3.77	381	422	142	159	128	126	402 ^{b}	151 ^{a}	127 ^{a}	**2.66**	**3.17**	1.19	**<.001**	0.856	0.944
	Dehydrotomatine	42	31.98	1030.5249	III	4	1.09	2.01	1093	1118	457	447	441	500	1106 ^{b}	452 ^{a}	470 ^{a}	**2.45**	**2.35**	-1.04	**<.001**	0.821	0.494
	alpha Tomatin	43	33.11	1032.5400	I	1,2	2.22	1.51	7009	7230	3426	3514	3121	3094	7119 ^{b}	3470 ^{a}	3108 ^{a}	**2.05**	**2.29**	1.12	**<.001**	0.903	0.971
	Acetoxytomatine + FA	44	33.17	1136.5511	IV	4	1.94	1.50	262	285	120	141	121	123	274 ^{b}	131 ^{a}	122 ^{a}	**2.09**	**2.25**	1.07	**<.001**	0.698	0.766
	alphaTomatin	45	33.62	1032.5397	I	1,2	1.71	1.15	532	598	170	170	139	136	565 ^{b}	170 ^{a}	138 ^{a}	**3.33**	**4.10**	1.23	**<.001**	0.853	0.744
	betatomatine	46	32.76	900.4974	II	2	0.37	1.35	846	1011	125	133	54	57	929 ^{c}	129 ^{b}	56 ^{a}	**7.20**	**16.63**	**2.31**	**<.001**	0.673	0.869
	Tomatin	47	32.60	1032.5391	III			1.13	165	186	33	44	41	38	175 ^{b}	39 ^{a}	40 ^{a}	**4.55**	**4.43**	-1.03	**<.001**	0.765	0.596
Flavonoid	Kaempferol dihexoside	48	23.02	609.1450	II	3	0.01	1.89	165	529	4044	4533	6051	5555	347 ^{a}	4288 ^{b}	5803 ^{b}	**-12.36**	**-16.72**	-1.35	**<.001**	0.183	0.094
	Quercetin 3 O-rutinoside	49	24.61	609.1453	I	1,2	0.40	1.90	483	1262	346	684	537	608	873	515	573	1.69	1.52	-1.11	0.079	0.106	0.212
	Kaempferol 3 O-rutinoside	50	27.64	593.1509	I	1,2	0.48	0.53	1214	1650	923	1110	1373	1603	1432	1016	1488	1.41	-1.04	-1.46	0.025	0.445	0.983
	Flavonoid 1	51	30.18	287.0559	III	5		0.67	211	285	145	175	266	235	248 ^{b}	160 ^{a}	250 ^{b}	1.55	-1.01	-1.56	**<.001**	0.378	0.083
Polyamine	Dicoumaroyl spermidine	52	19.42	436.2244	III	5		1.37	349	396	129	142	137	126	373 ^{b}	136 ^{a}	131 ^{a}	**2.75**	**2.84**	1.03	**<.001**	0.763	0.67
	Dicoumaroyl spermidine	53	20.24	436.2232	III	5		1.36	237	346	118	118	102	80	292	118	91	**2.47**	**3.21**	1.30	0.035	0.847	0.536
	Dicoumaroyl spermidine	54	20.55	436.2235	III	5		0.80	1173	1318	499	373	417	390	1245 ^{b}	436 ^{a}	403 ^{a}	**2.85**	**3.09**	1.08	**<.001**	0.767	0.114
	Dicoumaroyl spermidine	55	21.03	436.2240	III	5		0.46	688	1044	318	305	289	293	866 ^{b}	311 ^{a}	291 ^{a}	**2.78**	**2.98**	1.07	**<.001**	0.404	0.084
	Feruloyl coumaroyl spermidine	56	21.40	466.2347	III	7		15.76	179	243	56	52	52	54	211 ^{b}	54 ^{a}	53 ^{a}	**3.90**	**3.99**	1.02	**<.001**	0.57	0.592

Supplementary data Table 1 . *Cont.*

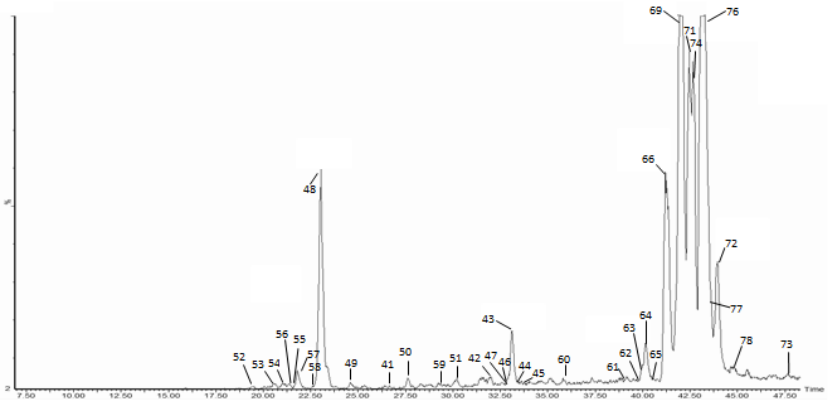
Polyamine	Dicoumaroyl spermidine	57	21.79	436.2235	III	5	0.80	3273	4672	1543	1285	1387	1319	3973 ^{b}	1414 ^{a}	1353 ^{a}	**2.81**	**2.94**	1.04	**<.001**	0.727	0.037
	Feruloyl coumaroyl spermidine	58	22.66	466.2360	III	7	12.89	420	596	173	141	140	138	508 ^{b}	157 ^{a}	139 ^{a}	**3.24**	**3.66**	1.13	**<.001**	0.753	0.028
	Tricoumaroyl spermine	59	29.26	639.3196	III	5	2.10	179	207	134	99	126	119	193	116	123	1.66	1.57	-1.05	0.022	0.727	0.326
	Tricoumaroyl spermidine	60	35.82	582.2622	II	6	2.79	174	184	95	95	110	84	179 ^{b}	95 ^{a}	97 ^{a}	1.89	1.84	-1.02	**<.001**	0.585	0.029
	Caffèoyl dicoumaroyl spermidine	61	38.90	598.2542	II	6	2.22	174	170	155	132	97	89	172 ^{b}	144 ^{b}	93 ^{a}	1.20	1.85	1.55	**0.002**	0.519	0.899
	Caffèoyl dicoumaroyl spermidine	62	39.52	598.2561	II	6	1.04	233	244	228	177	135	131	239 ^{b}	203 ^{b}	133 ^{a}	1.18	1.80	1.53	**<.001**	0.47	0.372
	Caffèoyl dicoumaroyl spermidine	63	40.09	598.2551	II	6	0.59	765	857	712	555	409	421	811 ^{b}	634 ^{b}	415 ^{a}	1.28	1.95	1.53	**0.003**	0.723	0.409
	Tricoumaroyl spermidine	64	40.16	582.2608	II	6	0.38	1427	1456	1413	2042	1710	1616	1442	1727	1663	-1.20	-1.15	1.04	0.342	0.717	0.264
	Caffèoyl dicoumaroyl spermidine	65	40.53	598.2556	II	6	0.12	294	343	264	196	172	165	319 ^{b}	230 ^{ab}	169 ^{a}	1.38	1.89	1.37	**0.007**	0.673	0.373
	Tricoumaroyl spermidine	66	41.22	582.2609	II	6	0.59	5275	5558	4879	5413	5059	4826	5417	5146	4943	1.05	1.10	1.04	0.244	0.816	0.338
	Dicoumaroyl spermidine	67	41.23	436.2245	II	5	1.57	158	159	147	165	154	143	159	156	149	1.02	1.07	1.05	0.242	0.93	0.085
	Feruloyl dicoumaroyl spermidine	68	41.65	612.2712	II	6	0.16	907	972	731	821	789	758	939 ^{b}	776 ^{a}	773 ^{a}	1.21	1.21	1.00	**0.008**	0.782	0.386
	Tricoumaroyl spermidine	69	42.00	582.2568	II	6	6.46	3545	3582	3308	3188	3370	3224	3564	3248	3297	1.10	1.08	-1.01	0.013	0.436	0.542
	Feruloyl dicoumaroyl spermidine	70	42.34	612.2712	II	6	0.16	2245	2403	1640	1571	1784	1507	2324 ^{b}	1605 ^{a}	1646 ^{a}	1.45	1.41	-1.03	**<.001**	0.495	0.039
	Tricoumaroyl spermidine	71	42.55	582.2566	II	6	6.88	11426	11645	9298	9268	9892	9009	11535 ^{b}	9283 ^{a}	9450 ^{a}	1.24	1.22	-1.02	**<.001**	0.644	0.278
	Feruloyl dicoumaroyl spermidine	72	43.95	612.2712	II	6	0.16	10474	11281	7829	6627	7715	7015	10877 ^{b}	7228 ^{a}	7365 ^{a}	1.50	1.48	-1.02	**0.004**	0.613	0.421
	tetracoumaroyl spermine	73	47.65	785.3560	II	6	0.95	384	399	317	228	313	275	392	272	294	1.44	1.33	-1.08	0.038	0.35	0.339
	Tricoumaroyl spermidine	74	42.57	582.2607	II	6	0.28	11426	11645	9298	9268	9892	9009	11535 ^{b}	9283 ^{a}	9450 ^{a}	1.24	1.22	-1.02	**<.001**	0.644	0.278
	Feruloyl dicoumaroyl spermidine	75	42.82	612.2709	II	6	0.33	1941	2053	1528	1449	1555	1438	1997 ^{b}	1488 ^{a}	1497 ^{a}	1.34	1.33	-1.01	**<.001**	0.77	0.358
	Tricoumaroyl spermidine	76	43.21	582.2566	II	6	6.88	3459	3609	3441	3429	3485	3488	3534	3435	3487	1.03	1.01	-1.01	0.265	0.564	0.326
	Feruloyl dicoumaroyl spermidine	77	43.48	612.2712	II	6	0.16	605	623	463	479	534	482	614 ^{b}	471 ^{a}	508 ^{a}	1.30	1.21	-1.08	**<.001**	0.87	0.314
	Diferuloyl coumaroyl spermidine	78	44.73	642.2816	III	7	0.18	652	696	446	370	419	388	674 ^{b}	408 ^{a}	403 ^{a}	1.65	1.67	1.01	**0.003**	0.723	0.493

Supplementary data Table 2 Statistic output of photodiode array data. The average per stage was calculated for each metabolite (from 6mm to M). Letters show statistically significant differences between the developmental stages per metabolite. Similar letters per metabolite indicate that there was no significant difference between the stages. Differences were considered statistically significant when the p-value of the ANOVA test was lower than 0.01 and the p-value of the Bonferroni post hoc test was lower than 0.05. Boxes represent p-values of the ANOVA test lower than 0.01 6mm, polarized microspore; 8mm, early bicellular pollen; M, mature; C, control condition; HS, short heat stress condition; Cor, corrected; Stag, developmental stages; Con, condition; Int, interaction stages*conditions

Class	Compound	6mm	8mm	M	Fold change 6mm vs 8mm	Fold change 6mm vs M	Fold change 8mm vs M	ANOVA p-value Stag.	ANOVA p-value Con.	ANOVA p-value Int.
Flavonoid	Kaempferol dihexose	6917 [a]	130483 [b]	176980 [b]	-18.86	-25.59	-1.36	**<.001**	0.271	0.234
	Quercetin 3-O-rutinoside	5790	4069	4094	1.42	1.41	-1.01	0.326	0.107	0.110
	Kaempferol 3-O-rutinoside	7381	5404	7814	1.37	-1.06	-1.45	0.058	0.334	0.862
	Flavonoid 1	2558 [b]	1680 [a]	2949 [b]	1.52	-1.15	-1.76	**<.001**	0.239	**0.005**
	Total flavonoids	22646 [a]	141637 [b]	191837 [b]	-6.25	-8.47	-1.35	**<.001**	0.020	**0.009**
Polyamine	Caffeoyl dicoumaroyl spermidine	2039 [b]	1599 [b]	986 [a]	1.27	2.07	1.62	**0.003**	0.447	0.766
	Dicoumaroyl spermidine	96303 [b]	41058 [a]	39552 [a]	2.35	2.43	1.04	**<.001**	0.751	0.044
	Diferuloyl coumaroyl spermidine	3182 [b]	1767 [a]	1890 [a]	1.80	1.68	-1.07	**0.003**	0.734	0.444
	Feruloyl coumaroyl spermidine	5845 [b]	646 [a]	294 [a]	9.05	19.87	2.19	**<.001**	0.677	0.664
	Feruloyl dicoumaroyl	194739 [b]	133751 [a]	139702 [a]	1.46	1.39	-1.04	**<.001**	0.649	0.265
	Tetra coumaroyl spermine	2843	2414	2853	1.18	-1.00	-1.18	0.706	0.529	0.251
	Tricoumaroyl spermidine	3567551 [b]	2253724 [a]	2389364 [a]	1.58	1.49	-1.06	**<.001**	0.576	0.297
	Tricoumaroyl spermine	1490	880	851	1.69	1.75	1.03	0.011	0.060	0.177
	Total polyamines	3873992 [b]	2435837 [a]	2575490 [a]	1.59	1.50	-1.06	**<.001**	0.600	0.269

Supplementary data table 3. Fold changes of compounds that showed a statistically significant difference at the two way interaction stage x condition (p-value of the ANOVA < 0.01). Stars indicate statistically significant fold change at the Bonferroni post hoc test. 6mm, polarized microspore; 8mm, early bicellular pollen; M, mature; C, control condition; HS, short heat stress condition

Compound	6mm-C vs 8mm-C	6mm-C vs. M-C	8mm-C vs. M-C	6mm-C vs 6mm-HS	8mm-C vs 8mm-HS	M-C vs M-HS
Flavonoid 1	1.30	-1.67	-2.16	-1.66	-1.26	1.19
Total flavonoids	-9.25 *	-13.42 *	-1.45	-2.05 *	-1.06	1.08



Supplementary data Figure 1 Total ion count chromatogram of mature pollen obtained by LC-QTOF-MS negative mode. Peaks are labelled with compound numbers that can be found in supplementary data Table 1

Chapter 5

Screening for pollen tolerance to high temperatures in tomato

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Abstract

Among the abiotic stresses affecting plant reproduction, high temperature is one of the most prominent ones because it directly affects fruit set. So far, little attention has been paid to the investigation of the variation in high temperature tolerance among wild tomato germplasm. The objective of this study was to determine the tolerance of 17 different cultivated and wild tomato accessions to high temperature, using a pollen viability screening approach. Each of the 17 genotypes of tomato was analysed for their pollen quality under a 32˚C (day) / 26˚C (night) regime. The total number of pollen per flower and the fraction of viable pollen were recorded. The number of pollen per flower varied between 35,547 and 109,490 whereas the fraction of viable pollen varied between 0.03 and 0.71. No correlation was found between these two traits. However, the combination of these traits could provide the best reproductive capability under high temperature. In this study, thermo-tolerant (LA2854, LA1478 and LA0417) as well as thermo-sensitive (LA1719, LA1580, and SWEET4) genotypes have been identified. Those genotypes can be used as novel genetic resources to get more insight into pollen thermo-tolerance mechanisms and be included in breeding programs.

Key words: pollen viability, heat stress, high temperature, tomato, screening, breeding, tolerance

Introduction

High temperature is one of the major abiotic stresses affecting plant reproduction, and therefore, fruit set (Dane et al. 1991). Most commercial tomato genotypes are not tolerant to high temperatures. A rise of a few degrees above the optimum growing temperature will, consequently, lead to a decrease of fruit set. Optimum growing temperatures for tomato are between 18˚C and 25˚C (Hurd and Cooper 1970). The predicted increase of 1 to 3˚C during the twenty-first century (IPCC 2012) may lead to a decrease of tomato production. Developing tomato genotypes tolerant to high temperature may be a valuable strategy to cope with these environmental changes. Tolerance to high temperature is not an easy trait to improve due to its low heritability (Hanson et al. 2002, Hazra et al. 2009), possibly due to its sensitivity to other environmental factors such as humidity (Abdulbaki 1991). To determine the tolerance of a plant to high temperature different parameters (Wahid et al. 2007) can be recorded, such as cell membrane thermo-stability, photosynthetic activity, pollen viability and fruit set. Fruit set is the ultimate measure for the tolerance of a genotype to high temperature and has been shown to correlate with a decrease of pollen viability in tomato (Firon et al. 2006). In general, genotypes tolerant to high temperature maintain a higher level of pollen viability under high temperatures than sensitive genotypes (Dane et al. 1991). The use of pollen viability as a screening tool will provide valuable information about the male gametophytic tolerance of different tomato genotypes to high temperatures. It is expected that focus on one aspect of the heat tolerance mechanism, pollen, will have simpler genetics, compared to screening for fruit production, which is a much more complex trait, determined by many more factors, such as flower formation, male and female tolerance, fruit set and fruit development. This is much more difficult to dissect genetically.

Several studies have focused on the identification of tomato cultivars tolerant to high temperature (Abdulbaki 1991, Abdelmageed and Gruda 2009, Da Costa et al. 2011, Kartikeya et al. 2012, Kugblenu et al. 2013). However, due to domestication and intensive breeding, the cultivated tomato germplasm has a rather narrow genetic basis. In contrast, there is a much larger genetic diversity in related wild relatives of tomato (Viquez-Zamora et al. 2013) and, hence, these may form an alternative source of thermo-tolerant genotypes. To date, little attention has been paid to the investigation of the variation in high temperature tolerance among the wild tomato germplasm. The identification of tolerant wild accessions may provide an additional valuable resource to develop tolerant tomato genotypes through classical breeding.

The objective of this study was to determine the tolerance of different cultivated and wild tomato accessions to high temperature, using a pollen viability screening approach.

Materials and methods

Plant materials

17 different tomato genotypes were tested under heat stress conditions in this study. Six wild S.*pimpinellifolium* accessions: LA0417, LA1478, LA1719, LA1580, LA1584 and

LA2854 were obtained from the Tomato Genetics Resource Centre (TGRC). These accessions were selected based on the environmental temperature and the altitude of the geographic locations they were collected from (Figure 1). All the *S. pimpinellifolium* accessions originated from locations with low altitude and hot environmental conditions – maximum average day temperature 28°C to 31°C. Seven *S.lycopersicum* genotypes: CLN1621F, CL5915-93D4-1-0-3, CL1131-0-0-13-0-6, CLN475BC1F2-265-4-19, CLN65-349D5-2-0, CL5915-206D4-2-2-0-4 and CL5915-153D4-3-3-0 were obtained from the Asian Vegetable Research and Development Centre (AVRDC) where they were annotated as 'tolerant to heat stress'. *S.lycopersicum* varieties Saladette and Nagcarlang were obtained from Radboud University, Nijmegen, The Netherlands and were annotated 'tolerant to heat stress' in the database of TGRC. SWEET4 is an introgression line developed at Wageningen UR Plant Breeding, The Netherlands, from a cross between *S. lycopersicum* cv. Moneymaker and *Solanum chmielewskii* LA1028. This introgression line was selected for its general vigour. *S.lycopersicum* variety M-82 obtained from TGRC was used as thermo-sensitive control.

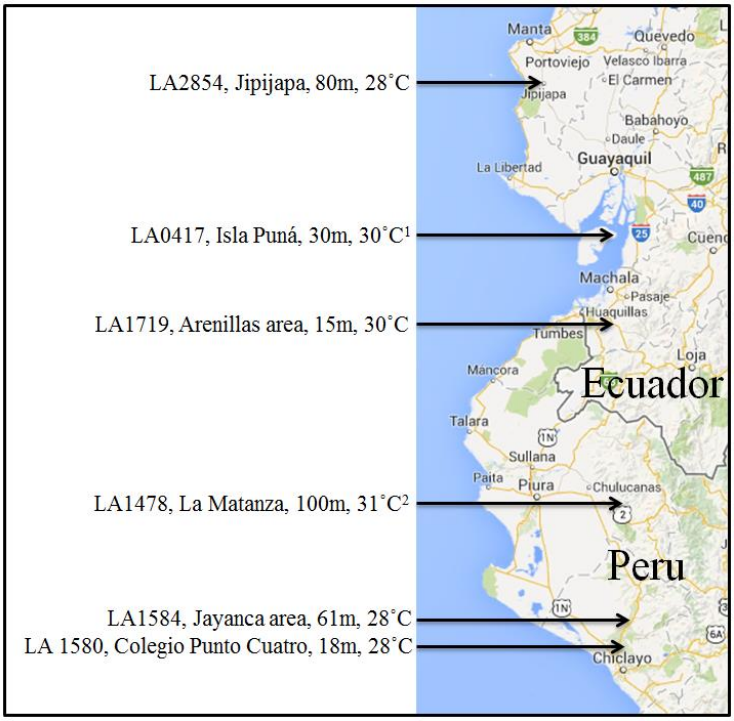

Figure 3. Geographical origins of S. pimpinellifolium accessions. X,X,X,X represents accession number from TGRC, site of collection, metres above sea level, average temperature. [1]Data temperature of Guayaquil, Ecuador. [2]Data temperature of Chulucanas, Peru (http://en.climate-data.org/)

Experimental growth conditions

The experiment was conducted in two periods. In the first period (March 2013), eight genotypes (CLN1621F, CL5915-93D4-1-0-3, CL1131-0-0-13-0-6, CLN475BC1F2-265-4-19,

CLN65-349D5-2-0, CL5915-206D4-2-2-0-4, CL5915-153D4-3-3-0 and M-82) were used and in the second period (April 2013), nine additional genotypes were used (LA2854, LA1478, LA0417, LA1584, LA1580, LA1719, Nagcarlang, Saladette and SWEET4). Plants were grown in the greenhouse of Unifarm (Wageningen University & Research Centre, The Netherlands) at 25˚C day and 19˚C night under 12 to 18 hours of natural day light for one month (Control condition). When the first flowers appeared, plants were moved and placed into a $15m^2$ climate chamber using a randomized design. The climate chamber was conditioned at 32˚C/26˚C, with 12 hours of day light provided with fluorescent tubes at a photon flux density of 500 µmol and 60% humidity. All flowers and buds from each plant were removed with forceps to assure that new flowers have been completely developed under the climate chamber conditions. Two weeks were needed to produce new open flowers.

Pollen viability test

For the pollen viability screening analysis, newly opened flowers were collected each morning from 9 to 11 am under the heat stress. In total, ten flowers per plant were collected and six plants were used per genotype. Flowers were collected into a petri dish filled with a wet paper. From each flower, anthers were cut into three to four pieces with a razor blade on a glass and put in an Eppendorf tube of 1.5mL . Then 0.5 mL of germination solution consisting of 1 mM KNO_3, 3 mM Ca $(NO_3)2 \bullet 4H_2O$, 0.8 mM $MgSO_4 \bullet 7\ H_2O$, 1, 6 mM H_3BO_3 was added to the Eppendorf tube (Adapted from Pressman et al. 2002, followed by 20 µL of Alexander dye. The Alexander dye consisted of 10 mL of 95% alcohol, 1 mL of malachite green (1% solution in 95% alcohol), 54.5 mL of distilled water, 25 mL of glycerol, 5 mL of Acid fuchsin (1% solution in water), 0.5mL of Orange G (1% solution in water) and 4 mL of glacial acetic acid for a 100 mL solution (Alexander 1980, Peterson et al. 2010). The Eppendorf tube was then vortexed for ten seconds and kept overnight at room temperature. The day after, pollen number was counted using a Fuchs-Rosenthal haemocytometer (W. Schreck Hofheim/Ts). 10µL of pollen solution was loaded into the haemocytometer and the number of viable pollen (stained purple) and non-viable pollen (stained green) were counted in eight squares of the haemocytometer for each sample using a light microscope. The counted number of pollen was then transformed in number of pollen per flower using the following formula:

$$\text{Number of pollen per flower} = \text{Number of pollen per square} * 2500^{a}$$

a was calculated based on the fact that each square contained 0.2µL of pollen solution, and the pollen of one flower was isolated in 500µL. Hence to determine the number of pollen per flower, the number of pollen per square was multiplied by 2500.

Pollen number counting for the first part of the experiment was performed in May 2013 whereas pollen number counting for the second part of the experiment was performed in July 2013.

Statistical analysis

Six biological replicates were used for each of the genotypes tested except for LA1478 for which four biological replicates were used. Each replicate represented a pool of pollen derived from ten flowers from the same plant. The fraction of viable pollen was determined by dividing

the number of viable pollen by the total number of pollen. Subsequently, an ANOVA test was performed together with a Tukey test. Genotypes from different growing periods were analysed independently. All analyses were done using SPSS 20ed.

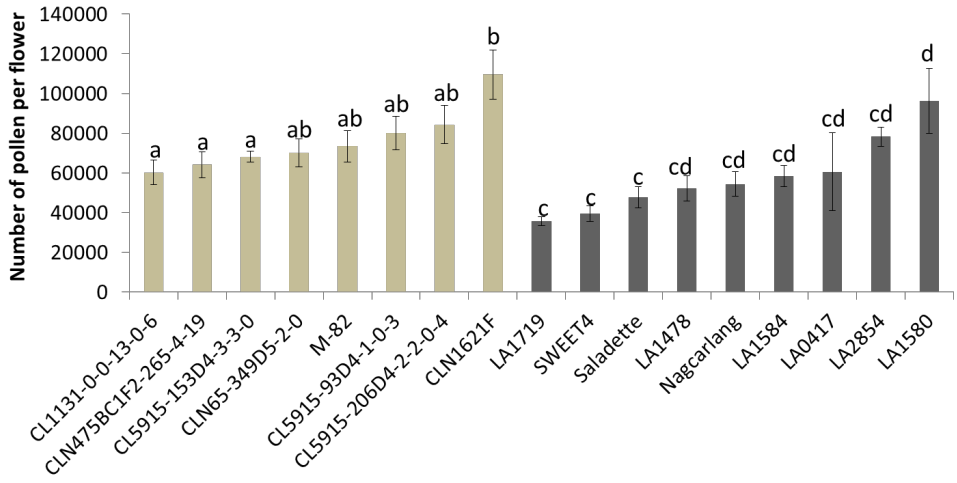

Figure 2. Number of pollen produced per flower per genotype under heat stress (32˚C/26˚C). Light bars represent genotypes grown in the first period whereas dark bars represent genotypes grown in the second period. Error bars represent standard error of the biological replicate mean. The letter above the bar represents the statistical difference based on a one way ANOVA with a Tukey's post-hoc test for the number of pollen per flower and per period. Statistics were performed independently for both periods. Genotypes with the same letter did not show statistically significant differences between each other (p-value > 0.05)

Results

Genotypic variation in total pollen production under heat stress

The total number of pollen produced under the high temperature (32˚C/26˚C) varied between the 17 accessions tested (Figure 2). Among the genotypes of the first period, the highest pollen number per flower was 109,490 (CLN1621F) and the lowest pollen number per flower was 60,266 (CL1131-0-0-13-0-6) under high temperature. The genotype CLN1621F showed significantly more pollen number per flower than the genotypes CL1131-0-0-13-0-6, CLN475BC1F2-265-4-19, CL5915-153D4-3-3-0. Among the genotypes of the second period, the highest pollen number per flower was 96,157 (LA1580) and the lowest pollen number per flower was 35,547 (LA1719) under high temperature. The genotype LA1580 showed significantly higher pollen number per flower than the genotypes LA1719, SWEET4 and Saladette. For instance, LA1580 produced two times more pollen than Saladette under high temperatures.

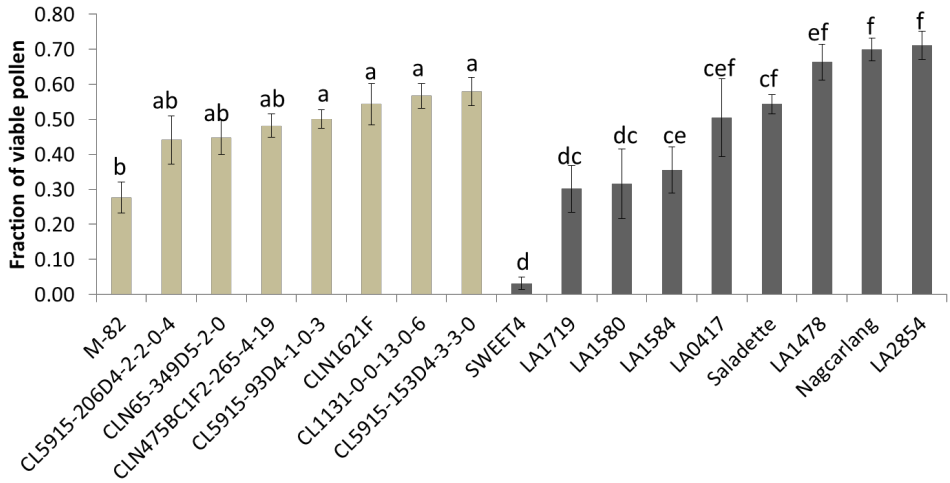

Figure 3. Fraction of viable pollen per genotype under heat stress (32˚C/26˚C). Light bars represent genotypes grown in the first period whereas dark bars represent genotypes grown in the second period. Error bars represent standard error of the biological replicate mean. The letter above the bar represents the statistical difference based on a one way ANOVA with a Tukey's post-hoc test for the fraction of viable pollen. Statistics were performed independently for both periods. Genotypes with the same letter did not have statistically significant differences between each other (p-value > 0.05)

Genotypic variation in pollen viability under heat stress

The fraction of viable pollen under the high temperature (32˚C/26˚C) varied between the 17 accessions tested (Figure 3). Among the genotypes of the first period, the highest ratio of viable pollen was 0.58 (CL5915-153D4-3-3-0) and the lowest ratio of viable pollen was 0.28 (M-82) under high temperature. The genotypes CL5915-153D4-3-3-0, CL1131-0-0-13-0-6, CLN1621F and CL5915-93D4-1-0-3 had a significantly higher ratio of viable pollen than M-82. In general, all seven genotypes obtained from AVRDC showed no statistically significant difference in pollen viability, which varied within 10% between them. More variation in pollen viability was observed in the second growing period. The highest ratio of viable pollen under the heat condition was 0.71 (LA2854) and the lowest ratio of viable pollen was 0.03 (SWEET4). The genotypes LA2854, Nagcarlang, LA1478, Saladette, LA0417 showed a significantly higher ratio of viable pollen than SWEET4, LA1719 and LA1580 under high temperature. There was no significant correlation observed between the total number of pollen and the viable fraction of pollen produced under high temperature (Figure 4). The Pearson correlation between these two traits is 0.19.

Based on their response to the two parameters tested, the genotypes can be considered at four different levels, with genotypes producing (i) a high total number of pollen with a high fraction of viable pollen (e.g. LA2854 and CLN1621F), (ii) a high total number of pollen with a low fraction of viable pollen (e.g. LA1580), (iii) a low total number of pollen with a high fraction

of viable pollen (e.g. Saladette), (iv) a low total number of pollen with a low fraction of viable pollen (e.g. SWEET4).

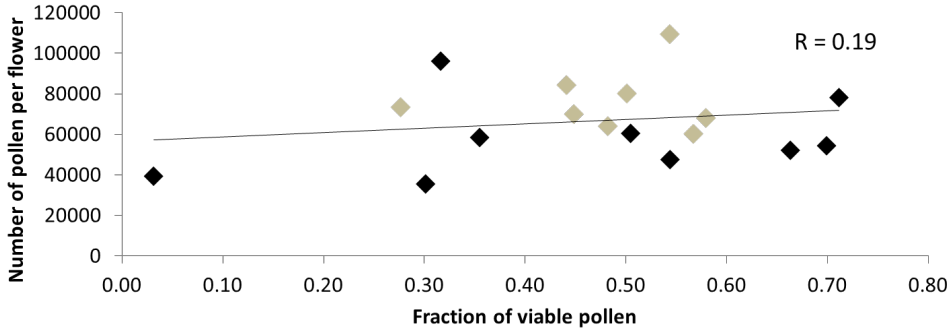

Figure 4. Correlation between the number of pollen per flower and the fraction of viable pollen produced under heat stress by different tomato genotypes tested. Light dots represent genotypes grown in the first period whereas dark dots represent genotypes grown in the second period. The correlation factor was calculated based on Pearson Correlation

Discussion

Wild tomato germplasm has been successfully used in breeding programs to introduce different traits, such as various disease resistance genes, into cultivated tomato (Labate and Robertson 2012). In this study we have selected six *S. pimpinellifolium* accessions, which originated from geographical regions with high average temperatures. All the selected *S. pimpinellifolium* accessions originated from a similar environment in terms of average maximum temperature ranging from 28˚C to 31˚C, which is higher than the optimal growing temperature for cultivated tomato (which is between 18˚C and 25˚C). These accessions were tested for total pollen number and pollen viability under high temperatures (32˚C during the day and 26˚C during the night) together with nine tomato genotypes, which have been reported to be tolerant to high temperature. Despite the similarity in their original growing environment, the *S. pimpinellifolium* accessions showed a high variation in both the total number of pollen and the fraction of viable pollen produced under high temperature. Among these six genotypes, three had a viable pollen fraction higher than 0.5. LA2854 and LA1478 belonged to the group with the highest fraction of viable pollen whereas LA1719 and LA1580 did not differ significantly from SWEET4 which had a pollen viability close to zero. This might suggest that different accessions may use different strategies to ensure a successful pollination in similar high temperature environments. This can be achieved e.g. by increasing the total number of pollen produced by a flower, by keeping the fraction of viable pollen high under high temperature, or a combination of both. Besides, although we did not assess the number of produced flowers, this trait might be of interest for the tomato production under high temperature.

The analysis of the two parameters ‘the total number of pollen produced per flower’ and ‘the fraction of viable pollen’ indicated that different genotypes vary in their response to high

temperatures. For example, accession LA1580 was among the best total pollen producers under high temperature, but did not belong to the group of genotypes with the highest fraction of viable pollen. Saladette belonged to the cluster with the highest fraction of viable pollen, but on the other hand it was one of the lowest total pollen producers. No significant correlation was found between the total number of pollen and the fraction of viable pollen. This suggests that the two parameters can represent independent pollen quality traits. Combining these traits could provide the best reproductive capability under high temperature. Considering these two parameters, *S. pimpinellifolium* LA2854, CLN1621F, and Nagcarlang, seem to be potentially the most heat tolerant accessions of the 17 genotypes tested, since they showed the highest fraction of viable pollen and a high total number of pollen. The analysis of pollen viability of the 17 genotypes was however performed only under high temperature, therefore we cannot exclude that under control conditions (e.g. optimal temperature for cultivated tomato) these genotypes may also show variation of these two parameters. Nevertheless, the ability to provide a good pollen quality under high temperature remains primordial to produce fruit.

Genotypes LA2854, Nagcarlang, CL5915-153D4-3-3-0, and CL1131-0-0-13-0-6, CLN1621F and CL5915-93D4-1-0-3 were the best genotypes regarding the fraction of viable pollen (from 0.50 to 0.71). A high fraction of viable pollen, in general, is correlated with a high fruit set in tomato (Firon et al. 2006). Recently, Kartikeya et al. 2012 analysed the percentage of fruit set relative to the number of total flowers and the percentage of viable pollen among 36 tomato varieties grown under high temperature and a high positive correlation between fruit set and pollen viability was observed. Based on this observation, we can speculate that the five best genotypes for the fraction of viable pollen may also have a high fruit set under high temperatures.

Indeed, some of the tested genotypes with a fraction of viable pollen higher than 0.5, such as CLN1621F, CL5915-93D4-1-0-3 and Saladette have already been shown to be tolerant to high temperatures in other studies (Da Costa et al. 2011, Lin et al. 2010, Abdulbaki 1991). For example, CLN1621F produced a high number of fruits per plant under high temperature, but these fruits generally had a small fruit weight (Da Costa et al. 2011). Tolerance to high temperature has indeed been shown to negatively correlate with fruit size (Wessel-Beaver and Scott 1992). The line CL5915-93D4-1-0-3 has already been used as a heat tolerant parent in a mapping population to identify quantitative trait loci (QTL) related to fruit quality, seed set and Brix under high temperature (Lin et al. 2010). Additionally, our results confirmed that M-82 is sensitive to high temperature with a fraction of pollen viability of 0.27. These results are consistent with those of Mazzeo et al. 2010. Although the environmental conditions of the two screening experiments we performed were kept identical, they were performed during two different adjacent time periods. Therefore, we cannot exclude that the growing period could have an effect on the total pollen production and pollen viability. Nevertheless, this study allowed us to discriminate between genotypes with a high pollen viability and genotypes with a low pollen viability.

Pollen development is one of the most sensitive processes in plants (Dane et al. 1991) and pollen quality is a major determinant of fruit production under high temperature. Several mechanistic studies have been conducted to get more insight in pollen thermo-tolerance

mechanisms. These studies focussed on different aspects of pollen development, such as pollen germination (Firon et al. 2006), thermo-sensitivity of pollen developmental stages (Sato et al. 2002), as well as monitoring temperature-induced changes in transcriptomic and proteomic profiles (Frank et al. 2009, Jagadish et al. 2010a (in anthers)). The identification of tomato genotypes with high pollen viability under high temperature conditions not only forms a valuable resource to study pollen thermo-tolerance mechanisms, but also helps to understand the underlying genetics and to breed for thermo-tolerance. Recently, a QTL study of pollen viability under high temperature has been carried out using a mapping population derived from CLN 1621L (tolerant) and CA4 (sensitive) tomato genotypes (Kardivel 2010). In this study, QTL LOD scores for pollen viability were low, suggesting a high complexity of this trait, where, besides genetic, many other factors may play a role. This is in line with the low heritability of tolerance to high temperature observed previously in tomato from a cross of CL5915-93D4-1-0-3 (tolerant) with UC204A (sensitive) (Hanson et al. 2002) and from cross combinations between CLN 2413R, CLN 2116B and COML CR-7 (tolerant) with the cultivars Patharkuchi and Ratan (sensitive) (Hazra et al. 2009). In agreement with the fact that other environmental factors than high temperature may play a role in the tolerance to high temperature, we noticed that the viable pollen fraction of tomato plants under high temperature may be variable regarding the time of the year in which the plants were grown before their transfer to the climate chamber (data not shown). These observations underlined the fact that other environmental factors influence the effect of high temperature stress on pollen viability.

Unravelling the genetics of a complex trait like pollen thermo-tolerance requires good insight in the mechanisms underlying pollen thermo-tolerance, in order to dissect this complex trait into sub-traits with simpler genetics. Furthermore, the success of such genetic studies is dependent on the availability of well characterised plant materials displaying variation in different parameters contributing to thermo-tolerance. Therefore, every new accession tolerant to high temperatures deserves to be studied for thermo-tolerance in detail. In this study, new thermo-tolerant (LA2854, LA1478 and LA0417) as well as thermo-sensitive (LA1719, LA1580, and SWEET4) genotypes have been identified. These can be used as novel genetic resources to get more insight into pollen thermo-tolerance mechanisms.

Acknowledgments

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Chapter 6

Metabolites associated with thermo-tolerance of tomato pollen

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Abstract

Developing pollen of tomato (*Solanum lycopersicum)* are sensitive to high temperatures, which leads to a reduction of fruit set when plants are cultivated under this type of stress. The decrease in pollen viability under high temperatures is strongly associated with metabolite alterations in anthers and pollen. In order to obtain a more comprehensive overview of metabolites associated with thermo-tolerance of the male reproductive organs we assessed the primary and secondary metabolomes of anthers of genotypes showing contrasting levels of pollen thermo-tolerance, M82 and Nagcarlang. Our results showed that under high temperatures the pollen viability of the more sensitive genotype M82 started to decrease during microsporogenesis and reached a minimum level at polarized microspore stage. The metabolic analysis of the anther development revealed that impairment of metabolism around that stage might explain the loss of fertility in M82 under high temperatures compared with Nagcarlang. The putative metabolites associated with this phenotype were the hexose sugars fructose and glucose, the amino acid proline, specific flavonoid glycosides, several conjugated polyamines and alkaloids. The role of these metabolites in providing a better thermo-tolerance to the male reproductive organs is discussed.

Key words: Anther development, metabolomics, untargeted analysis, heat stress, high temperature, tolerance

Introduction

Pollen grains are the male gametophyte, which develop inside the anther locules within the flower. At early developmental stages, the developing microspores receive nutrition via the tapetum cells that surround the anther locules (Pacini et al. 2006). During the polarization of the microspore, the tapetum degenerates and the locular fluid surrounding the developing pollen grains becomes the main nutritional source for the pollen grains (Pressman et al. 2012). One of the most studied metabolic pathways in the male reproductive tissue is the sugar metabolism. For instance, during the development of tomato pollen (*Solanum lycopersicum*), sucrose follows a gradient from the anther wall to the pollen grain, and represents 80% of the total soluble sugars in mature pollen grains (Pressman et al. 2012). The accumulated sugars are then used during pollen germination (Stanley 1971). Hence, their accumulation is pivotal to ensure proper fertilization. However, the tapetum cells are extremely sensitive to abiotic stresses (Parish et al. 2012). Although there is no data from tomato, in several plant species high temperatures lead to an early degeneration of the tapetum and shrinkage of the cells that might have a direct effect on the pollen nutrition. Under optimal growing conditions (28°C/22°C day/night), starch accumulation in developing tomato pollen reached a maximum three days before anthesis followed by a decrease until a minimum level at anthesis, while in the anther walls the starch concentration gradually decreased upon pollen maturation (Pressman et al. 2002). This decrease of starch concentration correlated with an increase of soluble sugars in both tissues. However, under high temperatures (32°C/26°C), the pollen grains did not accumulate starch which led to a strong decrease of soluble sugars in both anther walls and pollen grains. This metabolic impairment was accompanied by a reduction in pollen fertility (Pressman et al. 2002). Tomato genotypes that are tolerant to high temperatures regarding pollen development have been shown to maintain a higher level of sugars in pollen compared to sensitive genotypes (Firon et al. 2006). An exploration of the primary metabolome of mature anthers of rice genotypes with contrasting tolerance to heat and drought stress also suggested that the sugar metabolism is a key factor in maintaining fertility under changing temperatures (Li et al. 2015). Although great attention has been paid to sugar metabolism, other metabolites such as flavonoids and polyamines also play an important role in the development of a mature and fertile pollen grain (Paupière et al. 2014). The ability of the anther to maintain metabolic homeostasis during abiotic stress is likely to be one of the main factors affecting the final quality of the mature pollen. Hence, the analysis of the metabolic composition of the whole anther tissue may provide a greater understanding of the physiological basis of decreased pollen fertility under elevated growth temperatures.

The sensitivity of pollen to high temperatures varies over the course of their development. It is thought that meiosis and the microsporogenesis are the critical developmental stages with respect to high temperature tolerance (Muller and Rieu 2016). Iwahori (1965) demonstrated that in tomato, the most sensitive stage of pollen development to several hours of extreme heat of 40˚C was meiosis. Despite the high sensitivity of the meiosis stage to high temperatures, Pressman et al. 2002 did not observe an alteration of soluble sugars content at this stage under a long-term mild heat stress whereas the relatively tolerant mature stage showed a strong decrease in sugar content. Thus, it seems possible that under milder heat stress, later stages of

tomato pollen development, that accumulate essential metabolic resources, are most critical for the production of fertile pollen. Indeed, the microspore stage was identified as most sensitive to long-term mildly elevated growth temperatures in cowpea (*Vigna unguiculata*) (Ahmed et al. 1992). The current available studies on metabolites associated with temperature tolerance of the male reproductive tissue of tomato are limited and often focused on specific compounds (Paupière et al. 2014). This narrows down the possibilities to identify metabolites associated with heat stress tolerance and hampers our understanding of the complex biochemical processes of the stress response and the genetic factors that control them. A combination of untargeted metabolic profiling approaches such as Gas Chromatography coupled to Mass Spectrometry (GC-MS) and Liquid Chromatography coupled to Mass Spectrometry (LC-MS) may provide a better insight in the biochemistry of heat tolerance and make it possible to identify metabolic processes associated with thermo-tolerance in tomato.

We have previously identified tomato genotypes tolerant and sensitive to mild high temperatures (Chapter 5 of this thesis). In the present study, the whole anther metabolome of these genotypes was assessed under control and mildly elevated temperatures. Specific objectives of this study were (i) to characterize viability of developing pollen at different stages under control and high temperatures (ii) to explore the primary and secondary metabolome of anthers at different developmental stages under the two temperature regimes and (iii) to identify anther metabolites associated with high temperature tolerance of tomato.

Materials and methods

Plant materials and growth for pollen viability test

The *Solanum lycopersicum* tomato variety Nagcarlang was obtained from Radboud University, Nijmegen, The Netherlands and the genotype M82 from TGRC. M82 was previously identified as a high-temperature sensitive genotype regarding pollen viability and Nagcarlang was identified as a tolerant genotype (Chapter 5). The genotypes M82 and Nagcarlang were sown in September 2014 and grown in the greenhouse (Unifarm, Wageningen University & Research, The Netherlands) at 25˚C day and 19˚C night temperatures under 12 to 18 hours of natural day light for one month (control condition). When flowers appeared on plants, all the flowers and buds were removed and for each genotype eight plants were transferred in both, the control climate chamber (25˚C day and 19˚C night) and the heat stress chamber (precooled at 25˚C/19˚C). The following day, the temperature of the heat stress chamber was raised to 32˚C/26˚C, with a three hour period at 34˚C in the middle of the day (heat condition) to mimic natural daily temperature fluctuations. Light was provided for 12 hours per day with fluorescent tubes at a photon flux density of 500 µmol and 60% relative humidity. Two days after transfer into the growth chambers, plants of both chambers were sprayed with Mesurol® and Vertimec® to prevent thrips and spider mite infestation.

Pollen viability test

Pollen was harvested three to four weeks after plants were transferred to the climate chambers. Ten opened flowers per plant were harvested, and flowers of two plants were pooled to reach 20 opened flowers per biological replicate. Flowers were collected into Petri dishes on ice. From each flower, stigmas were removed, anthers cut into two pieces and transferred into a 50-mL falcon tube on ice. 10mL of cold germination solution (1 mM KNO_3, 3 mM Ca $(NO_3)2 \cdot 4H_2O$, 0.8 mM $MgSO_4 \cdot 7\ H_2O$, 1.6 mM H_3BO_3) was added (Adapted from (Pressman et al. 2002). Anthers were gently squeezed against the tube wall to promote pollen release. The sample was vortexed for ten seconds and the liquid was filtered through two layers of miracloth Calbiochem® and transferred into a new falcon tube on ice. The sample was centrifuged at 300 rpm for 15 minutes at 4˚C. Supernatant was discarded and the pollen pellet was washed with 500 µL of cold germination. 20 µL of pollen solution was diluted with 500 µL of cold germination solution and 20 µL of Alexander dye was added to stain the pollen grains. The Alexander dye consisted of 10 mL of 95% alcohol, 1 mL of malachite green (1% solution in 95% alcohol), 54.5 mL of distilled water, 25 mL of glycerol, 5 mL of Acid fuchsin (1% solution in water), 0.5mL of Orange G (1% solution in water) and 4 mL of glacial acetic acid for 100 mL solution (Alexander 1980), (Peterson et al. 2010). The sample was then vortexed for ten seconds and kept overnight at room temperature. Pollen viability counting was assessed as previously described in Chapter 5.

Experimental growth conditions for anther metabolome analyse

To explore the anther metabolome of M82 and Nagcarlang genotypes under control and heat stress, 20 plants per genotype were sown in December 2015 and grown under control conditions. After two months, plants were moved to climate chambers with control and high temperature conditions as previously described. For each treatment ten plants per genotype were used and a biological replicate was considered as a pool of two plants. After one month, the pollen developmental stages were assessed and anthers were harvested for metabolomics analysis.

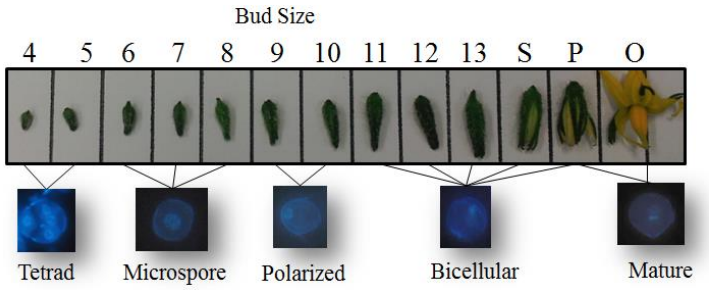


Figure 1. Relation between bud size and pollen developmental stage in tomato. Pollen were stained with DAPI and observed under a fluorescent microscope.

Pollen development determination

To determine pollen developmental stages of M82 and Nagcarlang flowers under control and heat stress, six flowers of different sizes were randomly harvested for each condition per genotype. Buds with sepals still closed were grouped by size from 4 mm to 13 mm, and buds with opened sepals were grouped in three categories: S, opened sepals with closed petals; P, opened sepals with opened petals; and F, fully opened flowers (Figure 1). Bud size was determined with the whole flower from the length of the anther tip (including sepal) to the base of the anther. Petals and sepals were removed. Buds of the same size were pooled and put into a 1.5-mL Eppendorf tube with 500 µL of ice cold germination solution. Anthers were gently squeezed against the Eppendorf tube wall before to be vortexed. 5 µL of pollen solution was mixed with 5 µL of DAPI staining solution were loaded on a slide and observed using a fluorescent microscope. Pollen were scored according to their developmental stages: tetrad, microspore, polarized microspore, bicellular pollen and mature pollen.

Harvest of developmental stages of anthers

Anthers of M82 and Nagcarlang at different developmental stages were harvested for metabolomics analysis. The harvest was spread over four days with each day representing a condition per genotype. Buds were organized by sizes and stages on ice. Different numbers of buds were used per biological replicate: 30 for tetrad stage, 26 for microspore, 12 for polarized microspore and eight for bicellular pollen and mature pollen. Stages were obtained by pooling different bud sizes, dependent on genotype and treatment. For Nagcarlang under control conditions, 4 mm buds were harvested for tetrad stage; 5-6 mm for microspore; 7 mm for polarized microspore; 8-9 mm and S for bicellular pollen. For Nagcarlang under heat stress, 6 mm bud corresponded to tetrad stage; 7 mm for microspore; and 9-10 mm for bicellular pollen. For M82 under control conditions, 4-5 mm corresponded to tetrad stage; 6-8 mm for microspore; 9-10 mm for polarized microspore; 13 mm and S for bicellular pollen. For M82 under heat stress, 6 mm buds were harvested for tetrad stage; 7-9 for microspore; 10-12 for polarized microspore; S for bicellular. Open flowers were used for mature pollen stages for each condition and genotype. Petals and sepals were removed from buds and flowers of 5 mm to F, and only sepals were removed from buds of 4 mm. Pistil was removed from each bud and flower. Anther cones were transferred into a 2-mL Eppendorf tube frozen in liquid nitrogen and stored at -80˚C. Frozen anthers were then grinded with a pestle and mortar in liquid nitrogen, and weighed before being used for metabolite extraction.

Metabolite extraction

Extraction of polar and semi-polar metabolites from anther and pollen materials was carried out at room temperature. Extraction of samples was done randomly and divided over three days. To each sample 350 µL of 100% methanol containing ribitol as internal standard and 150 µL of distilled water were added. Samples were sonicated for 15 min followed by a centrifugation at 17,000 g for ten minutes. The supernatant was used for orbitrap LC-MS and GC-MS analyses.

For LC-MS orbitrap, 200 µL of the methanol-supernatant was centrifuged at 17,000 g for ten minutes. The extract was then filtered over a 0.2 µm polytetrafluoroethylene (PFTE)

filter. 100 µL of filtered extract was transferred into a 2 mL crimp glass vial with insert for LC-MS analysis.

For GC-MS, 250 µL of the methanol-supernatant was mixed with 250 µL of distilled water and 125 µL of chloroform. The sample was vortexed for five minutes and centrifuged at 17,000g for ten minutes. 40 µL of the supernatant was transferred into a crimp vial with insert and dried overnight in a centrifugal evaporator until complete dryness before GC-MS analysis.

Additionally to the experimental samples, quality control samples (QCs) were produced for the metabolomics platforms. These QCs consisted of aliquots of a pool of the final extracts. These QCs were used to estimate the technical variation of the analytical platforms, including extract analysis and untargeted data processing.

Metabolic profiling

Polar primary metabolites of anthers were analysed with an Optic 3 high-performance injector (ATAS) and an Agilent 6890 gas chromatograph coupled to a Pegasus III TOF mass spectrometer (Leco) as previously described by Carreno-Quintero et al. 2012. Dried extracts were derivatized using an on-line automatic procedure performed by a Combi PAL autosampler (CTC analytics). 12.5µL of O-methylhydroxylamine hydrochloride (20 mg mL−1 pyridine) was added to the samples and incubated for 30 min at 40°C. Then, the samples were derivatized with 17.5µL of N-methyl-N-trimethylsilyltrifluoroacetamide (TMS) for 30 min. Then 5 µL of alkane mixture was added and the derivatization process continued for an additional 30 min. For measurement, 2 µL of extract was introduced to the injector at an initial temperature of 70°C in a split injection mode (vent flow 40 mL/min; split flow 19 mL/min). The injector was heated with 6°C s^{-1} to 240°C. The chromatographic separation was performed using a VF-5ms capillary column (Varian; 30 m × 0.25 mm × 0.25 µm) including a 10-m guardian column with helium as carrier gas at a column flow rate of 1 mL min^{-1}. The temperature was isothermal for 2 min at 70°C, followed by a 10°C min^{-1} ramp to 310°C, and was held at this temperature for 5 min. The transfer line temperature was set at 270°C. The column effluent was ionized by electron impact at 70 eV. Mass spectra were acquired at 20 scans s^{-1} within a mass-to-charge ratio range of 50 to 600 at a source temperature of 200°C. A solvent delay of 295 s was set. The detector voltage was set to 2000V. The data were recorded with ChromaTOF software 2.0.

Semi-polar secondary metabolites of anthers were analysed with a LTQ orbitrap LC-MS (Thermo Fisher Scientific) using a C18 column (Phenomenex), an Accela HPLC tower connected to a photodiode array (PDA) detector and an LTQ/obritrap hybrid mass spectrometer as previously described by van der Hooft et al. 2012 and by Moco et al. 2006. Mass spectrometry was performed using a negative ionization mode. For the measurements, 10 µL of sample was injected into the system. The Xcalibur software was used for data acquisition.

Metabolomics data processing

Both GC-MS and LC-MS data were processed using MetAlign software (www.metalign.nl; (Lommen 2009)), to correct for baseline, peak picking and mass alignment of chromatograms as previously described by Tikunov et al. 2005 and De Vos et al. 2007, respectively.

For GC-MS, the MetAlign output was reduced by omitting mass data showing peak intensity values lower than the detection threshold, i.e. >125 in more than five samples. Masses below 85 Da were deleted due to their low specificity. Mass spectra and quantitative ions of compounds detected were extracted from the modified MetAlign outputs using MSClust software (www.metalign.nl) (Tikunov et al. 2012). The MSClust output was normalised by ribitol abundance for values higher than the detection threshold to correct for batch effect and then normalised by sample weight.

For LC-MS, the mass peaks were extracted and aligned by MetAlign software. MSClust software (Tikunov et al. 2012) was then used to group masses originating from the same molecule and extract quantitative ions of compounds detected. If a quantitative ion selected by MSClust showed saturation of the MS detector, this ion was replaced by its second or third isotopic ion. Data were normalised by sample weight.

Compound annotation

The MSClust outputs of GC-MS and LC-MS were used for compound annotation. Annotation of compounds detected by LC-MS was performed using an in-house metabolite database generated by previous experiments on tomato tissues (e.g. Moco et al. 2006, van der Hooft et al. 2012) and online databases such as METLIN (http://metlin.scripps.edu/). The annotation of compounds was performed according to The Metabolomics Standards Initiative requirements (Sumner et al. 2007): identified compounds got level I when Nuclear Magnetic Resonance was performed or an authentic standard has been used for unambiguous identification, level II when no authentic standard was used but annotation was made with both physicochemical property and spectral similarities, and level III when the (class of the) compound has previously been reported for tomato, and finally level IV in case further annotation of detected metabolite was impossible. The definition of unknown compounds was based on the mass clustering determined by MSClust, fragmentation and adduct information. Annotation of GC-MS compounds was done through automatic matching of the mass spectra generated by MSClust with the spectra provided by the National Institute of Standards and Technology (NIST) and the Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de/) using MS Search software (NIST). Compound annotation was based on both mass spectra matching and the difference in retention index between the library entries and the experiment data. Compound annotation for primary metabolites and secondary metabolites are listed in Supplementary data table S1 and table S2, respectively.

Statistical analysis

Most of the statistical analyses were performed with SPSS software, except when mentioned otherwise. For the pollen viability data, a univariate ANOVA was performed followed by a

post-hoc Tukey test (α=0.05) to correct for multiple pairwise comparisons. For the metabolomics data, the principal components analysis (PCA) was performed with Genemaths software (http://www.applied-maths.com/) on log2 transformed and mean-centred values. To identify statistical differences between samples a univariate ANOVA analysis was performed for each annotated metabolite on log2 transformed values with the following model

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + \delta + \varepsilon_{ijk},$$

where μ = overall mean , α_i = main effect of conditions *i*, β_j = main effect of genotypes *j*, γ_k = main effect of developmental stages *k* , $(\alpha\beta)_{ij}$ = interaction between conditions *i* and genotypes *j* , $(\alpha\gamma)_{ik}$ = interaction between conditions *i* and developmental stages *k*, $(\beta\gamma)_{jk}$ = interaction between genotypes *j* and developmental stages *k*, $(\alpha\beta\gamma)_{ijk}$ = three-way interaction between conditions *I*, genotypes *j* and developmental stages *k*, δ = random effect of observations within plants, ε_{ijk} = random error term. The random effect was introduced in order to overcome the dependency between stages, since the samples at the five developmental stages were taken from the same plant. A significance threshold of 0.001 was chosen to account for the large number of variables (99 annotated metabolites) tested. Metabolites with a p-value lower than 0.001 were then followed by a post-hoc Bonferroni test (at α=0.05) to correct for multiple pairwise comparisons among treatment combinations.

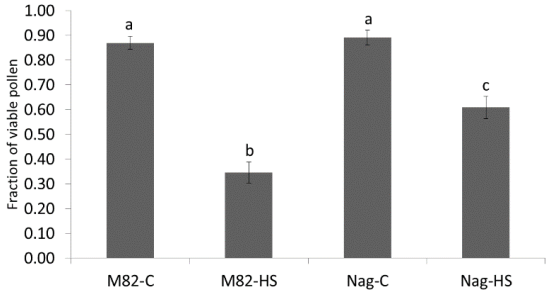


Figure 2. Viability of mature pollen of M82 and Nagcarlang under control and heat stress conditions. Nag, Nagcarlang; C, control condition; HS, heat stress condition.

Results

Genotypic variation in pollen viability under heat stress

Since the pollen viability of Nagcarlang and M82 was previously assessed during two different periods of time (Chapter 5), the pollen viability screening was repeated with both genotypes growing at the same time. Under control conditions, pollen viability of M82 and Nagcarlang were comparable - about 87% for both genotypes. Under the heat conditions Nagcarlang showed a statistically significant ($p<0.05$) higher percentage of viable pollen (60%) than M82 (30%) (Figure 2), which was comparable to the viability ratio reported in Chapter 5.

Development of M82 and Nagcarlang pollen under heat

To study the effect of heat on the morphological development of pollen, flower buds were collected from M82 and Nagcarlang plants at control conditions and at heat stress conditions. Buds of each variety were grouped by average size, ranging from 4 mm to open flower and the composition of pollen developmental stage present within each group was then determined for each of the 13 size categories. Five pollen development stages were discriminated (from young to mature): tetrad, microspore, polarized microspore, bicellular pollen and mature pollen (Figure 1). The transition between pollen developmental stages was not fully synchronised and appeared as a gradual change in the ratio between the developmental stages within different bud size groups (Figure 3). Pollen stage counting started in that bud size group, in which clear tetrads became visible. Hence, the minimum bud size at which pollen stages were counted differed depending on the different combinations of genotype and condition. The developmental stages were determined among viable cells. With DAPI staining a cell was considered viable if it was roundly shaped and not shrunken.

In M82 under control conditions, 4 and 5 mm buds contained developing pollen at tetrad stage (Figure 3). Microspore stage pollen constituted 86-98% of 6 to 8 mm buds and were still present in 9-10 mm buds (25-32%), along with polarized microspores (53-67%) and 6-15% of bicellular pollen. The number of bicellular pollen then increased to a maximum of 79% in 13 mm buds and 18% of them were still present in open flowers, whose anthers consisted for 82% of fully mature pollen. The viability of M82 pollen during development under control

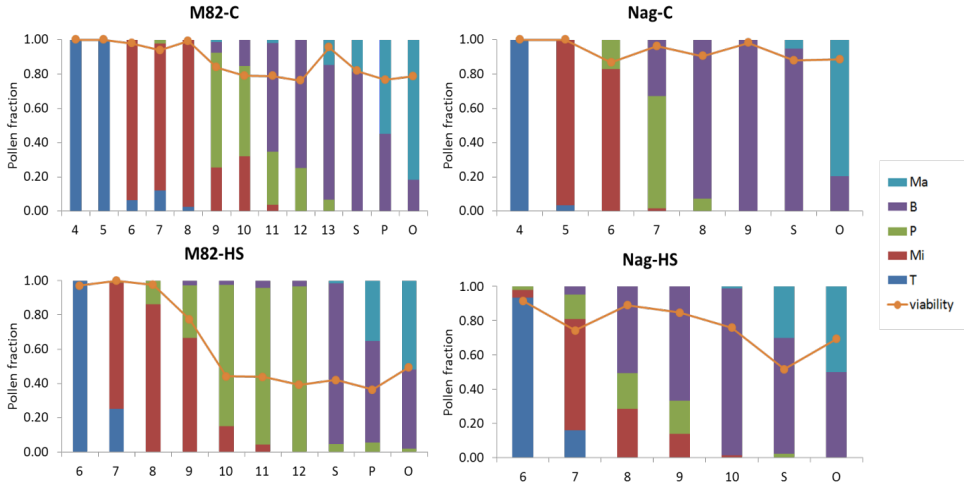


Figure 3. Pollen development profile and pollen viability per bud size. Nag, Nagcarlang; C, control condition; HS, heat stress condition; T, tetrad stage; Mi, microspore stage; P, polarized stage; B, bicellular stage; Ma, mature stage. From 4 to 13 are the bud sizes in mm; with S, sepal open; P, petal open and O, complete open flower.

conditions showed a slight decrease, but still was about 80% in open flowers. The maximum length of Nagcarlang buds before sepal opening was 9 mm, thus, 4 mm shorter than in M82. This explains why the pollen stage ratios observed in Nagcarlang buds and flowers at the control

condition were shifted towards shorter bud sizes. As with M82, the viability of Nagcarlang pollen did not change considerably during development at control condition and was about 90% in open flower. For both cultivars, the pollen stage ratios changed under heat conditions. All the development stages were observed in larger buds compared to the control conditions, indicating decoupling of developmental progression and floral organ growth under the heat condition. For example, 6 mm buds of M82 still fully consisted of tetrads at heat condition whereas at control condition buds of the same size had more than 90% of the developmentally more advanced microspores. Similarly, 11 and 12 mm buds of M82 had about 91-97% polarized microspores at heat, while in buds of equal size at control condition their number already decreased to 25-31% and they were mostly developed into bicellular pollen. A similar heat induced delay in pollen development relative to flower size could be observed in Nagcarlang buds. Interestingly, the number of polarized microspores in Nagcarlang buds of any of the sizes never exceeded 21%, whereas at control condition they constituted about 66% of the content of 7 mm buds. In contrast, 10-12 mm buds of M82 accumulated up to 83-97% of polarized microspores at the heat condition, compared to 25-53% in M82 buds of similar size under control conditions.

In both genotypes, a decrease in pollen viability was observed under heat conditions. Under control condition, all developmental stages of M82 and Nagcarlang showed high pollen viability. At the heat condition, however, viability dropped in M82 by 22%, already in 9 mm buds – the bud size where also the percentage of polarized pollen began to increase quickly (Figure 3). The viability in M82 continued to decline to 44% in 10 mm buds and then remained at this level until flowers opened. In Nagcarlang the decrease of pollen viability was more gradual and reached 70% at maturity.

Thus, the pollen development process occurs in a genotype-dependent manner, both under control and under heat conditions relative to the bud size. The heat condition led to a 2-fold stronger decrease in viability of M82 pollen compared to Nagcarlang, which seemed to correlate with a slower nucleus polarization process observed in M82.

Metabolic composition depends on developmental stages rather than condition

To study how heat stress affects the metabolic composition of tomato pollen and its surrounding tissue and how this differs between genotypes with contrasting pollen thermo-tolerance levels, we analysed whole anthers of the two tomato genotypes M82 and Nagcarlang. Anthers of M82 and Nagcarlang, containing particular pollen developmental stages, were collected from flowers developed under control or heat stress conditions and their composition of primary and secondary metabolites was analysed by GC-MS and LC-MS, respectively. The average abundance of each metabolite per sample is shown in Supplementary data table 1 and 2.

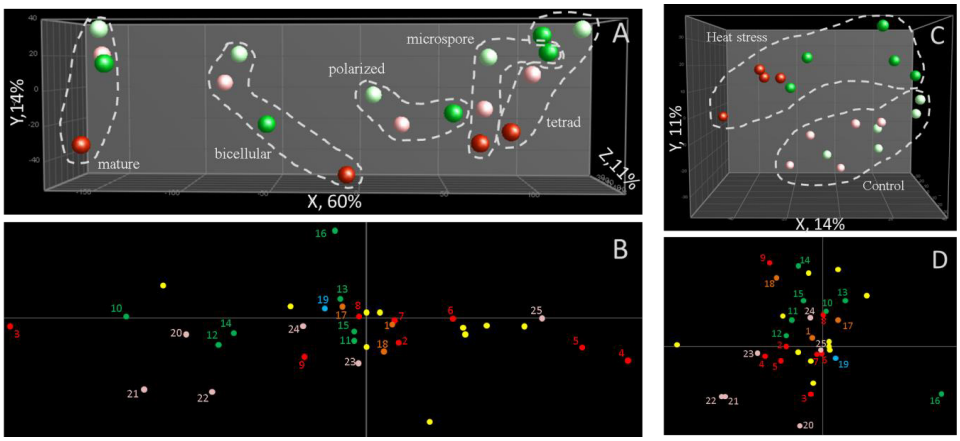


Figure 4. Principal component analysis of the primary metabolome (PCA). PCA plots A and B represent the PCA with component 1,2 and 3 which are the axes X, Y and Z, respectively. The PCA plots C and D represent the PCA with component 2, 3 and 4 which are the axes X, Y and Z, respectively. The PCA plots A and C represent the distribution of samples. Light green, M82 control condition; dark green M82 heat stress condition; pink, Nagcarlang control; red, Nagcarlang heat stress. The PCA B and D represent the distribution of metabolites responsible for the variation of samples in PCA A. With red dots, amino acid; green, organic acid; blue, phenolic acid; light pink, sugars; orange, other class and yellow unknown. The metabolite numbers to the annotation are provided in Supplementary data table 1.

Primary polar metabolites

A Principal Components Analysis (PCA) was performed with the 36 primary metabolites detected and showed that most of the variation present among the samples (Principal Component 1, PC1=60%) was due to the metabolic differences between the five developmental stages: tetrad, microspore, polarized, bicellular and mature (Figure 4A). On the one hand, most of the amino acids were more abundant in the younger developmental stages than in mature stages, except for proline which was 28-fold higher in mature stage than in tetrad stage (Figure 4B, Supplementary data table 3). On the other hand, the sugars and organic acids were less abundant in younger developmental stages than in the bicellular stage. For instance, fructose (Metabolite number 21) and malic acid (10) were 10-fold and 6-fold higher in bicellular stage than in tetrad stage, respectively (Supplementary data table 3). The heat stress treated Nagcarlang sample separated on the Y-axis (PC2) from the rest of the samples indicating a specific heat stress response in Nagcarlang (Figure 4A). In addition, analysis of PC2 and PC3, which represented 14% and 11% of the total variation, respectively, showed a clear heat stress effect on the anthers (Figure 4C). Most of the organic acids accumulated more in the heat stress condition, whereas the level of the specific organic acid glucaric acid (16) was 3.4-fold lower under control than under heat stress conditions (Figure 4D, Supplementary data table 3). Also the amino acid glutamine (9) accumulated more (3.2-fold) under heat stress than under control conditions (Supplementary data table 3). Hence, although most of the variation was due to developmental stages, the heat stress clearly affected the primary metabolome of anthers. Nagcarlang, which showed higher pollen viability under heat stress, also showed a metabolic response in this condition which was different from the response of the sensitive M82.

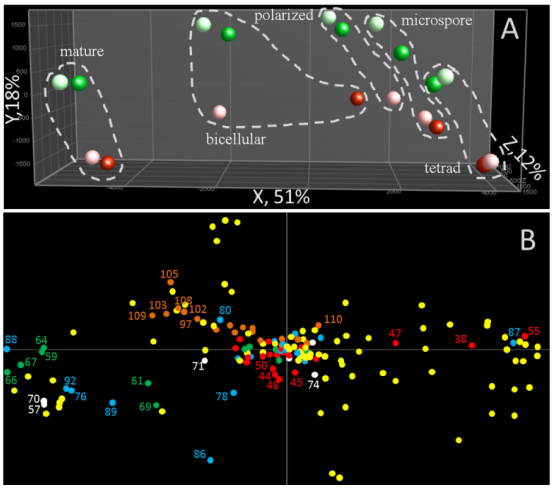


Figure 5. Principal component analysis of the secondary metabolome (PCA). The PCA plot A represents the distribution of samples with light green, M82 control condition; dark green M82 heat stress condition; pink, Nagcarlang control; red, Nagcarlang heat stress. The PCA plot B represents the distribution of metabolites responsible for the variation of samples in PCA A. Red dot, alkaloid; green, flavonoid; blue, phenolic acid; orange, polyamine; white, other class and yellow unknown. The metabolite number to the annotation in Supplementary data table 2.

Secondary semi-polar metabolites

Similar to the primary metabolites, the PCA performed on the secondary metabolites showed that the metabolic differences between the developmental stages represented most of the variation in the data (PC1=51%) (Figure 5A, B). Most of the flavonoids and phenolic acids were present at higher levels in later developmental stages than in polarized stages. For instance, kaempferol 3,7-di-O-glucoside (58) strongly accumulated from tetrad stage to mature stage (3.5-fold, Supplementary data table 3). It is also important to notice that a large number of unknown metabolites played a role in the separation of the developmental stages. Unlike what was observed for primary metabolites, the second principal component, which explained 18% of the variation among the samples, was due to metabolic differences between the two genotypes. The polyamines tended to accumulate more in M82 compared to Nagcarlang, e.g. dicoumaroyl putrescine (105) which was 2-fold higher in M82 than in Nagcarlang (Supplementary data table 3). PC5 and PC6 separated the control condition from the heat stress condition, but these components represented only a low percentage of the variation present within the data (PC5=3.2% and PC6=2.3%).

Identification of metabolites linked with tolerance

To determine which metabolites were associated with thermo-tolerance we decided to focus our statistical analyses on metabolites that showed contrasting patterns between the thermo-tolerant Nagcarlang and the thermo-sensitive M82 with a minimum difference of at least 2-fold.

Table 1. The table represents the metabolites that showed a specific heat stress response between the development stage per genotype (CxGXD). Crosses represent the statistical significance at the ANOVA test for the different factors tested (α=0.001).

Name	ANOVA						
	C	G	D	CxG	CxD	GxD	CxGxD
3_L-Proline	X	X	X		X	X	X
6_L-Aspartic acid			X	X	X	X	X
10_Malic acid	X	X	X		X	X	X
11_Threonic acid	X		X		X	X	X
12_2,3-Dihydroxybutanedioic acid	X		X		X	X	X
15_Galactonic acid	X		X		X		X
21_Fructose		X	X		X	X	X
22_Glucose		X	X		X		X
23_myo-Inositol	X	X	X		X	X	X
37_Hydroxytomatine I + FA	X	X	X	X	X	X	X
40_Dehydrotomatine FA	X	X	X		X	X	X
41_Dehydrotomatine FA isomer I		X	X			X	X
42_Dehydrotomatine FA isomer II	X	X	X		X	X	X
43_alpha-Tomatin + FA	X		X			X	X
44_Hydroxytomatine + FA isomer I		X	X		X	X	X
45_Hydroxytomatine + FA isomer II	X	X	X		X	X	X
49_Hydroxytomatine isomer I	X	X	X				X
55_beta-tomatine	X	X	X	X	X	X	X
58_Kaempferol 3,7-di-O-glucoside	X		X		X	X	X
60_Kaempferol 3-O-rutinoside	X	X	X		X	X	X
61_Narginin-hexoside, -deoxyhexoside	X		X		X		X
62_Kaempferol-hexoside-deoxyhexoside, -pentoside	X	X	X		X	X	X
63_Quercetin-hexoside-deoxyhexoside, -pentoside	X		X	X	X	X	X
66_Dihydrokaempferol-hexoside or Eriodictyol chalcone-hexoside 2	X		X	X	X	X	X
67_Kaempferol 3,7-di-O-glucoside isomer I	X	X	X	X	X	X	X
68_Kaempferol-Hexoside	X	X	X		X	X	X
74_Benzyl alcohol-hexoside-pentoside isomer II			X		X	X	X
81_Homovanillic acid-O-hexoside isomer I	X	X	X		X	X	X
82_Homovanillic acid-O-hexoside isomer II	X	X	X		X	X	X
91_Coumaroylquinic acid isomer	X	X	X		X	X	X
98_Dicoumaroylspermidine isomer II		X	X	X		X	X
102_Caffeoyl dicoumaroyl spermidine isomer II			X		X		X
105_Dicoumaroylputrescine	X	X	X		X	X	X
106_Dicoumaroylspermidine IV	X		X	X		X	X

C, condition; G, genotype; D, development; CxG and GxD, two-way interaction and CxGxD three-way interaction

We focused on those metabolites that: (i) showed a differential response to the heat stress between the genotypes at a specific developmental stage (GxCxD interaction, table 1). Among the 99 annotated metabolites, including primary and secondary metabolites, 34 showed a statistically significant interaction effect of the conditions, the developmental stages and the genotypes (p-value <0.001, Supplementary data table 4).

(ii) showed different developmental accumulation patterns between genotypes (GxD). In total 33 metabolites showed a significant difference at the interaction GxD excluding metabolites that appeared at the CxGxD interaction (Supplementary data table 6).

(iii) showed a genotype dependent response to the condition (GxC). In total eight metabolites showed a statistically significant difference at the interaction GxC excluding metabolites that appeared at the CxGxD interaction (Supplementary data table 5).

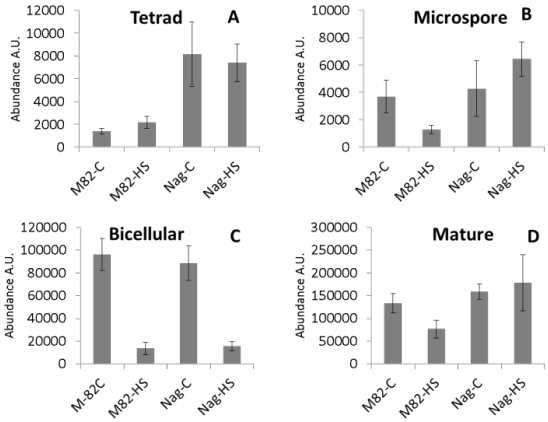


Figure 6. Abundance of the amino acid proline at different developmental stages under control and high temperatures for both genotypes. Nag, Nagcarlang; C, control condition; HS, heat stress condition. Error bar represents the standard deviation of the mean (n=4-5). Notice the different scales used in each panel.

Amino acids and organic acids

Of the eight detected amino acids, seven showed a significant difference in the ANOVA (p-value <0.001, Supplementary data table 1), but only the amino acid proline (3) showed a strong significant association with the heat stress condition (>2-fold, Supplementary data table 4). In general, proline accumulated during anther development in both M82 and Nagcarlang (Figure 6), but the initial level of proline at tetrad stage was higher in Nagcarlang compared to M82, irrespective of the heat condition (Figure 6A). Under heat stress condition the proline accumulation dynamics was strongly delayed, which resulted in about 7-fold lower abundance in the heat stressed bicellular pollen of both genotypes (Figure 7C), though this was much less obvious in mature pollen (Figure 4D). At microspore stage, which was identified as the most critical stage for pollen quality, the proline level in the heat-sensitive M82 was strongly decreased, by 2.9-fold, under heat stress compared with control condition, whereas no significant difference between the conditions was observed for Nagcarlang at this developmental stage (Figure 7B, Supplementary data table 4). As a result, Nagcarlang, whose pollen viability was not significantly affected under heat stress, accumulated 5-fold more proline than M82 at this specific stage. Interestingly, at microspore stage the biosynthetic precursor of proline, glutamic acid, was 1.5-fold less abundant in M82 than in Nagcarlang (Supplementary data table 6).

The amino acids serine (4) and β-alanine (5) showed similar quantitative patterns (Supplementary data table 1). Both amino acids were more abundant at the early – tetrad and microspore - stages, and their concentration decreases at later developmental stages in both genotypes. In Nagcarlang heat stress led to a dramatic increase of these amino acids at bicellular

stage, compared to the control condition, whereas in M82 they did not reveal a quantitative heat stress response at this developmental stage. Neither of the two compounds passed the significance threshold in the interactions with condition, most likely due to the presence of non-detectable levels in some replicates and the corresponding loss of statistical power.

None of the detected organic acids showed a clear difference in response to the heat stress between the two genotypes (Supplementary data table 1).

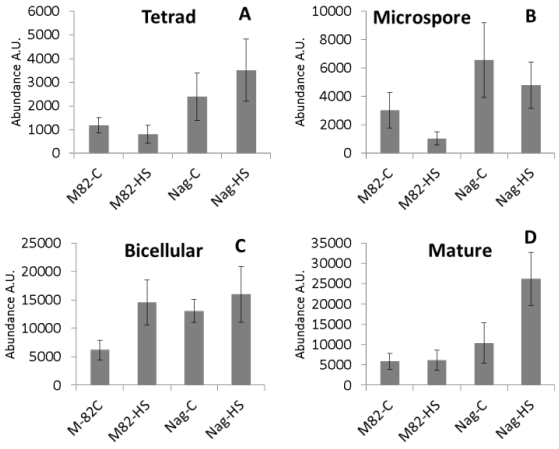
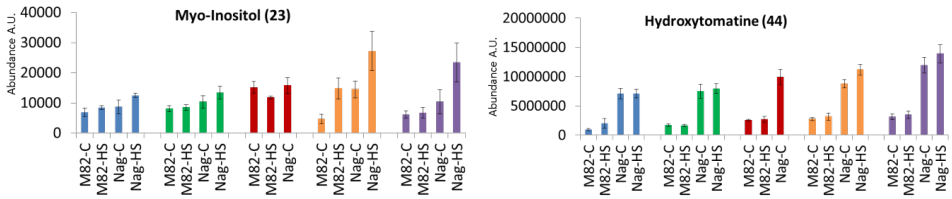


Figure 7. Abundance of glucose at different developmental stages under control and high temperatures for both genotypes. Nag, Nagcarlang; C, control condition; HS, heat stress condition. Error bar represents the standard deviation of the mean (n=4-5)

Sugars

Hexoses – glucose (22) and fructose (21) – showed overall higher basal abundance in anthers of Nagcarlang (Supplementary data table 1). For example, at the most sensitive microspore stage, Nagcarlang accumulated two-fold more glucose and fructose than M82, even in the control condition, although these differences did not reach our statistical threshold (Figure 7B, supplementary data table 4). Upon the heat stress, their abundance dropped in M82 anthers by 2.9-fold (for glucose) compared with control condition (Figure 7B, Supplementary data table 4). As a result, under heat stress condition, Nagcarlang accumulated up to 4.5-fold more hexoses than M82 at both early stages (Figure 7A and B, supplementary data table 4). Under heat stress condition, mature stage anthers of Nagcarlang accumulated up to 6-fold of the hexose sugars (Figure 7D) and almost 4-fold of the sugar alcohol myo-inositol (23) compared to M82 (Figure 8). No differences were found for their precursor, sucrose (Supplementary data table 1).



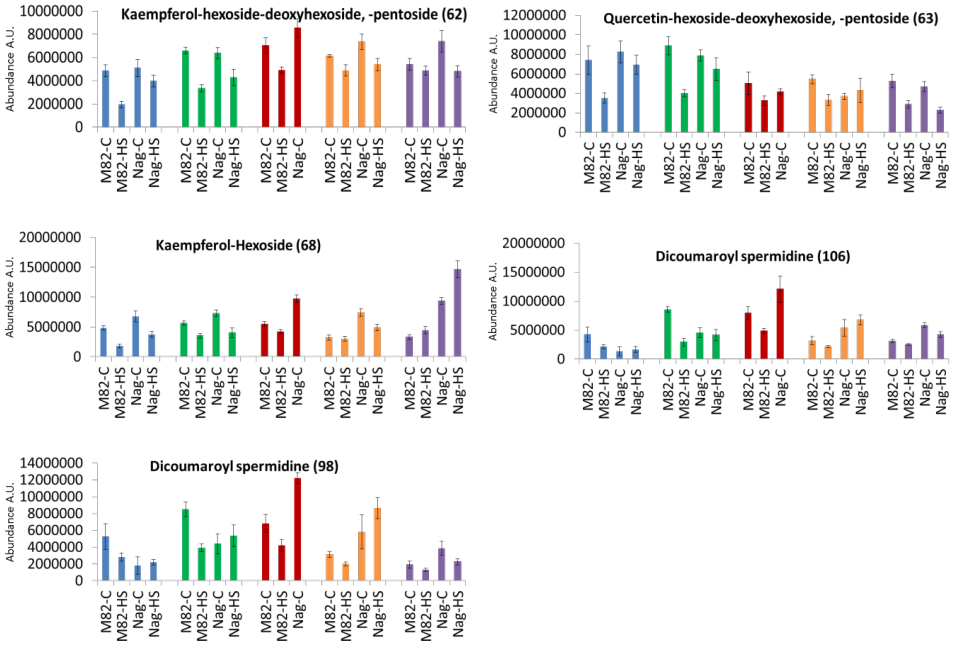


Figure 8. Abundances of selected metabolites that showed significant interaction (GxCxD) in the ANOVA ($p<0.001$). Blue bar, tetrad stage; green bar, microspore stage; red bar, polarized stage; orange bar, bicellular stage; purple bar, mature stage; Nag, Nagcarlang; C, control condition; HS, heat stress condition; T, tetrad stage; Mi, microspore stage; P, polarized stage; B, bicellular stage; Ma, mature stage. Error bar represents the standard deviation of the mean (n=4-5)

Alkaloids

Most of the detected alkaloids accumulated at higher levels in Nagcarlang compared to M82 under both control and heat stress conditions and did not show a different heat stress response between the genotypes (Supplementary data table 2 and 4). For instance, the hydroxytomatine isomer I (44), II (45), III (48) and IV (50) accumulated up to 14-fold more in Nagcarlang compared with M82 (Figure 8, Supplementary data table 4 and 6). A similar accumulation was also observed for leptinidine trihexoside (38), dehydrotomatine (40), and dehydrotomatine isomer I (41) and II (42), especially at young anther stages. Acetoxy-tomatine isomer II (46), however, accumulated at 2-fold higher levels in M82 compared to Nagcarlang at microspore stage (Figure 9, Supplementary data table 6). Interestingly, the precursors alpha-tomatin (43) and beta-tomatin (55) did not show a differential accumulation pattern between the two genotypes (Supplementary data table 4).

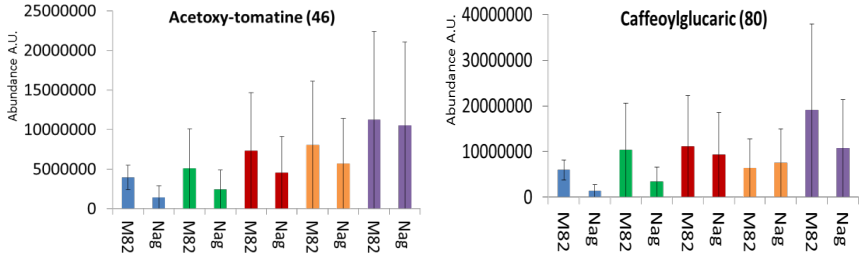


Figure 9. Abundances of selected metabolites that showed significant statistical differences at the GxD interaction in the ANOVA ($p<0.001$). The value represent the average per stage of both conditions. Blue bar, tetrad stage; green bar, microspore stage; red bar, polarized stage; orange bar, bicellular stage; purple bar, mature stage; Nag, Nagcarlang; C, control condition; HS, heat stress condition; T, tetrad stage; Mi, microspore stage; P, polarized stage; B, bicellular stage; Ma, mature stage. Error bar represents the standard deviation of the mean (n=5-10)

Flavonoids

At early stages, two flavonoid glycosides responded differently to the heat stress in the two genotypes: levels of kaempferol hexoside deoxyhexoside pentoside (62) and quercetin hexoside deoxyhexoside pentoside (63) decreased by 2-fold upon heat stress at tetrad and microspore stage in M82, while such a strong decrease was not observed in Nagcarlang (Figure 8, Supplementary data table 4). This difference in heat stress response resulted in a higher abundance of these two flavonoids at tetrad and microspore stage in Nagcarlang compared to M82 under heat stress. At all stages except mature pollen, kaempferol hexoside (68) levels decreased upon heat stress in both genotypes, but Nagcarlang accumulated 2.1-fold more of this flavonoid glycoside compared to M82 at tetrad stage under heat stress (Figure 8; Supplementary data table 4). In anthers at mature pollen stage, levels of this flavonoid were significantly higher in Nagcarlang compared to M82 and increased even more in Nagcarlang upon heat stress. Interestingly, during all stages of anther development, kaempferol 3-O-rutinoside-7-O-glucoside (69) accumulated to a higher level in Nagcarlang compared to M82, independent of the condition (up to 7-fold at microspore stage, Figure 10). In general, the other flavonoids decreased upon heat stress in both genotypes (e.g. kaempferol 3,7-di-O-glucoside and its isomer I (58, 67), kaempferol 3-O-rutinoside (60), but their levels were not different between the two genotypes (Supplementary data table 4).

Acids conjugated with hydroxycinnamic acid

Several acids conjugated with hydroxycinnamic acids showed a significant statistical difference in the ANOVA analysis, but many of these differences were less than 2-fold. In general, the most profound changes in the levels of these metabolites occurred at late anther developmental stages (Supplementary data table 1 and 2). For instance, levels of coumaroylquinic acid (75) accumulated 4.3-fold higher in Nagcarlang compared to M82 at bicellular stage, and feruloylquinic acid-O-hexoside isomer I (76) was 2.9-fold more abundant in M82 than in Nagcarlang at mature stage (Supplementary data table 6). The caffeoylglucaric acid isomer (80) was the only hydoxycinnamic acid conjugate showing a strong differential accumulation at early stages: up to 4.3-fold higher levels in M82 compared to Nagcarlang (Figure 9).

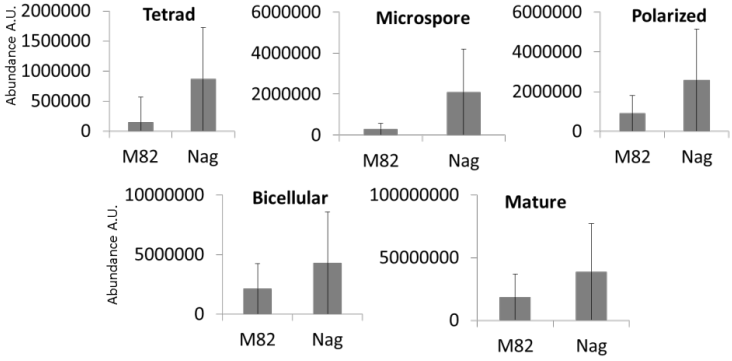


Figure 10. Abundance of the flavonoid kaempferol 3-O-Rutinoside-7-O-glucoside at different developmental stages for both genotypes. The values represent the average per stage of both conditions. Nag, Nagcarlang; Error bar represents the standard deviation of the mean (n=5-10).

Conjugated polyamines

In total 18 conjugated polyamine forms were detected. Three of them showed a genotype-dependent difference in abundance at microspore stage: upon heat stress dicoumaroyl spermidine isomer II (98), caffeoyl dicoumaroyl spermidine isomer II (102), and dicoumaroyl spermidine IV (106) decreased by 2.2, 6.6 and 3-fold in abundance, respectively, in M82 only (Figure 8, Supplementary data table 4), while the levels of these compounds in Nagcarlang did not change significantly upon heat stress. At tetrad stage, M82 accumulated feruloyl dicoumaroyl spermidine isomer III (108), which was not detectable in Nagcarlang at that stage. Nagcarlang started to accumulate this compound from microspore stage onwards, at which its abundance was still 2.3-fold lower than in M82. At later developmental stages, the abundance of this compound was similar in both genotypes (Supplementary data table 6).

Discussion

Metabolites are known to have a large diversity of functions. They can be used as energy source, e.g. sugars (Eveland and Jackson 2012), to build proteins, e.g. amino acids, to constitute a membrane, e.g. lipids (Quinn and Williams 1978), to protect against herbivores, e.g. alkaloids (Friedman 2002), and oxidative stress, e.g. flavonoids (Agati et al. 2012), and to serve as signal molecules, e.g. volatile methyl salicylate (Cojocariu et al. 2004). The homeostasis of metabolites is a fragile balance that can be disturbed by external parameters such as environmental stresses. In our study, we have shown that anthers of tomato which developed under high temperatures showed a decrease of pollen viability. The corresponding quantitative metabolic responses observed might be an acclimation response, by which the cells attempt to regain homeostasis or activate protection mechanisms, or a passive consequence of the changing physiology of the cells, which could include the underlying cause of the reduced pollen fertility. Further studies will be needed to demonstrate if there are functional relationships between the pollen fertility and the metabolic changes observed in our study. In the following part of our discussion, we will focus first on the transition between microspore stage and polarization, as a critical step of the developing pollen. Secondly, we will discuss how differences between the two genotypes in their metabolic response to stress might explain the better tolerance of Nagcarlang to high temperatures.

Microspore – polarization as a critical developmental step

In Chapter 5 of this thesis we have shown that Nagcarlang is a more tolerant genotype compared to M82 under mild heat stress. However, in that experiment the two genotypes were grown in two different periods of time, and we have mentioned that different growing periods can have a slight effect on the pollen quality of the flower (Chapter 5). In the present study, the two genotypes were grown at the same period of the year and our results confirmed that Nagcarlang performed better than M82 under high temperature. In order to determine at which stages the viability of the developing pollen dropped under high temperature and led to the low pollen viability at anthesis in M82, we have recorded the pollen viability during development.

In the middle 1960's meiosis was identified as a stage of pollen development which was the most sensitive to heat stress (Iwahori, 1965b). Since then, the number of studies on this topic have grown and as reviewed by Muller and Rieu 2016 different aspects of the pollen development have been demonstrated to be sensitive to high temperatures such as alteration in chromosome behaviour and cytoskeleton dynamics occurring during the meiosis, and a defect of microsporogenesis with an alteration of the tapetal cells. The tapetum is a key tissue that provides nutrition to the pollen at early stages before it degenerates when the microspore cell starts to vacuolate and form the polarized microspore (Sawhney and Bhadula 1988). Once the tapetum is degenerated the metabolites are delivered to the pollen from the locular fluid (Pressman et al. 2012). Interestingly we found that the pollen viability of the sensitive genotype started to decrease during microsporogenesis which is in line with observations in cowpea anthers developed under mild heat stress (Ahmed et al, 1992a). Furthermore, they observed that just after the release of the tetrad, the tapetum layer degenerated prematurely and suggested that this was the underlying cause of the cowpea sterility under high temperatures. It is tempting to speculate the same occurs in tomato and leads to a decrease of nutrition to the pollen. This idea is supported by our observations that several metabolites showed a drastic drop in accumulation rate during the microspore stage, specifically the sugars, recognised as the main energy resource for the pollen. At the microspore stage, tapetum is considered to be metabolically extremely active due to the increase of the number of mitochondria per cell (Parish et al. 2012, De Storme and Geelen 2014). The high number of tapetal mitochondria might increase the demand for sugars, which could lead to an unbalanced homeostasis under heat stress. Interestingly, at these specific stages the tolerant genotype Nagcarlang accumulated more sugars than the sensitive genotype M82, in particular under heat stress at which M82 was unable to maintain its sugar levels. Tolerant tomato genotypes have already been associated with higher levels of sugars (Firon et al. 2006). Hence, we can also hypothesise that the level of sugars present in M82 might be insufficient to cope with the higher demand for sugars from the tapetal cells, leading to starvation. Although in our case, the mature anthers did not show a difference in sugar levels between control and heat stress, it was previously demonstrated that, under similar mild heat stress condition, the final mature pollen of the sensitive genotype was sugar deficient (Firon et al. 2006). The question whether the decrease of pollen viability is due to an early degeneration of the tapetum which impairs the metabolism or to energy starvation still remains open and requires further studies.

Metabolites that promote the tolerance of Nagcarlang

We have shown that in our study the microsporogenesis is a critical step to maintain high pollen viability under a mild heat stress of 32°C-34°C during the day and 26°C during the night. Therefore, the metabolic profiles observed at this stage of development may help to find out the biochemical and physiological processes that cause the difference in tolerance between the two genotypes. We carried out two types of metabolomics analyses, and although most of the differences present among the samples were due to the developmental stages, we also found differences in the heat stress response and in the basal level of metabolite abundance. Studies on the effect of heat stress on the primary metabolome have already been performed in floral organs of rice (Li et al. 2015) and leaves of Bentgrass (Xu et al. 2013). This allowed the

identification of sugar metabolism, branched amino acids and proline as metabolite markers for tolerance, but the secondary metabolome remained unexplored. Here, we detail the specific metabolic signatures of different metabolic classes in relation to the difference in heat tolerance between M82 and Nagcarlang.

Amino acids

The amino acid proline is known to be the most abundant amino acid in anthers (Mutters et al. 1989). It has been demonstrated to be stress-responsive and associated with tolerance (Hayat et al. 2012). It is pivotal to the pollen viability of tomato (Mattioli et al. 2012). During the development of anthers under heat stress, the proline content of Nagcarlang was not affected by the heat stress and showed a higher accumulation compared to M82. In anthers of M82, the heat stress led to a reduction of proline content. Our observations slightly differ from those of Mutters et al. 1989 who demonstrated in cowpea a strong accumulation of proline in anther walls, and a decrease of proline in pollen, suggesting an inhibition of proline translocation from the anther wall to the pollen. While we did not study tissue-specific distribution, the overall reduction in level of proline in M82 compared with Nagcarlang might be associated to the inability to protect itself, since proline is known to be involved in (i) ROS scavenging, (ii) stabilizing protein and (iii) promoting a cell redox balance (Verbruggen and Hermans 2008). It is interresting to mention that proline is directly synthetised from the amino acid glutamate (Forde and Lea 2007). During the early development stages, Nagcarlang showed a higher level of glutamate than M82, which correlates with the higher level of proline. Therefore, we can speculate that the alteration of the proline level in early developmental stages in M82 anthers in response to heat stress might be partly due to differences in the metabolism of its precursor glutamate.

The two genotypes showed a strong difference in the accumulation of the amino acids serine and alanine at bicellular stage under heat stress. Since these differences occurred after the drop in pollen viability, it is most likely that they did not play a major role in the maintenance of microspore viability. However, we cannot exclude that these two amino acids might play a role in maintaining metabolite homeostasis under high temperatures at later developmental stages.

Sugars

Sugars are often considered as key metabolites in the maintenance of high pollen quality under heat stress (De Storme and Geelen 2014, Muller and Rieu 2016). De Storme and Geelen (2014) proposed that the decrease of pollen viability is due to reduction of sucrose utilization rather than to a decrease in sucrose availability under heat stress. As previously mentioned higher levels of sugars have been associated with temperature tolerance of reproductive organs (Firon et al. 2006, Li et al. 2015). They are used as energy source for the developing pollen (Obermeyer et al. 2013) and pollen tube growth, but also as osmolyte (Hare et al. 1998). In line with this, the tolerant genotype Nagcarlang accumulated more glucose and fructose and the sugar alcohol myo-inositol than M82 under heat stress. The ability to have a higher energy resource might be of great help to survive unfavourable conditions.

Flavonoids

Among the secondary metabolites, the flavonoids are well studied compounds in reproductive organs, since chalcone synthase mutants are sterile in many crops, as was demonstrated in maize (*Zea mays*), petunia (*Petunia hybrida*) (Mo et al. 1992) and later in tomato (Schijlen et al. 2007). Flavonoids are antioxidants that scavenge reactive oxygen species (ROS) (Agati et al. 2012). High temperatures are known to induce ROS production (Agati et al. 2012), and therefore a well running antioxidant system is crucial to avoid oxidative damage. As previously mentioned the anther tapetum cells of the microspore stage are metabolically very active as reflected by the extremely high number of mitochondria present in these cells (Parish et al. 2012). Since ROS are highly produced in the mitochondria, Muller and Rieu 2016 suggested that under high temperatures, these cells might be prone to accumulation of ROS. Under heat stress, Nagcarlang maintained a higher abundance of some flavonol glycosides, such as kaempferol-hexoside-deoxyhexoside, -pentoside (62), quercetin-hexoside-deoxyhexoside, -pentoside (63) and kaempferol-hexoside (68) compared with M82. Therefore, we can speculate that M82 has a less efficient antioxidant system to deal with high-temperature induced ROS accumulation during microsporogenesis and that this, in turn, leads to developmental failure.

Polyamines

Besides flavonoids, polyamines are also important secondary metabolites in anthers and pollen. The inhibition of polyamine synthesis by cycloheximide or RNAi-mediated down-regulation of the pathway genes led to a reduction of pollen viability (Chibi et al. 1993, Sinha and Rajam 2013). As recently reviewed by Tiburcio et al. 2014 and Aloisi et al. 2016, polyamines are involved in modelling the pollen cell wall, regulating the level of ROS and promoting pollen tube growth. Although depletion of polyamines led to deleterious effects, it has also been demonstrated that a high concentration of polyamines in the germination medium decreased the pollen fertility of Japanese apricot (*Prunus mume*) (Wolukau et al. 2004). For instance, the male sterile *stamenless-2* mutant showed a high content of polyamines that might be associated with an abnormal anther pattern (Rastogi and Sawhney 1990). Hence, a homeostatic balance of the polyamine metabolism is essential to ensure flower fertility. Although the PCA seemed to indicate that M82 accumulated a larger pool of polyamines, the statistical analysis revealed that most of the polyamines did not show a different level of polyamines, neither between the genotypes at different anther development stages, nor in response to heat stress. As for other metabolites discussed above, the specific caffeoyl dicoumaroyl spermidine isomer, dicoumaroyl spermidine IV and dicoumaroyl spermidine isomer II showed a decrease in abundance in the less tolerant M82 under heat stress at microspore stage. Therefore, it is tempting to make the association that higher level of these conjugated spermidine forms might be linked with the better tolerance of Nagcarlang but further experiments are necessary to confirm the specific role of these polyamines in the tolerance to high temperature.

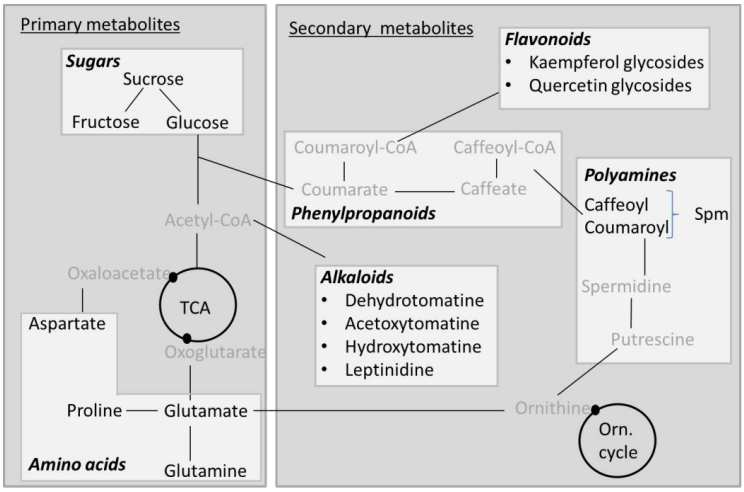


Figure 11. Metabolic network of compounds putatively associated with thermo-tolerance. Compounds in light grey were not detected. Spm, spermidine; Orn. Ornithine; TCA, tricarboxylic acid cycle

Alkaloids

The alkaloids are mainly known for their protective role against biotic stresses (Friedman 2002) and to our knowledge little attention has been paid to the involvement of these metabolites in the protection to temperature stresses. Interestingly, Nagcarlang accumulated a large amount of alkaloids under both control and heat stress conditions, compared to M82. Therefore, investigations are required to determine if high levels of alkaloids might help Nagcarlang to withstand high temperatures better than M-82.

Conclusions and perspectives

We have established a list of metabolites (Table 1) that might be associated with the thermo-tolerance of Nagcarlang and discussed their putative role in this process. Their position in a metabolic network are shown in Figure 11. Both primary and secondary metabolism were putatively associated with the thermo-tolerance, involving different groups of metabolites, including sugars, amino acids, flavonoids, polyamines and alkaloids. In agreement with the current literature, we have (i) confirmed that the sugars and the amino acid proline are associated with thermo-tolerance in reproductive organs (ii) proposed specific flavonoids and polyamines to contribute to the tolerance, and (iii) identified a new class of metabolites, the alkaloids, that might be involved in the tolerance. Further analysis of these metabolic thermo-tolerance markers needs to be assessed before they can be widely used in breeding strategies to develop tomato varieties more tolerant to high temperatures.

Acknowledgments This study was supported by SPOT-ITN, Solanaceae Pollen Thermo-tolerance - Initial Training Network (Grant agreement 289220). We thank all the members of SPOT-ITN and Ric de Vos for the fruitful discussions and Bert Schipper for the technical assistance on LC-QTOF-MS.

Supplementary data table 1. Average abundance of primary metabolites per sample. Values in Italic light grey are random values below the detection threshold. Ret, retention time; ΔRI, delta retention index; MF, match factor, . Cross in the ANOVA section represent metabolite that showed a statistical significant difference at the ANOVA analysis (p-value<0.001) for the factor condition (C), genotype (G), development (D), the interaction condition x genotype (CxG), the interaction condition x development (CxD), and the interaction condition x genotype x development (CxGxD); T, tetrad; Mi, Microspore; P, Polarized; B, Bicellular; Ma, Mature; Nag/N, Nagcarlang; C, control; HS/H, Heat stress; M, M82

Name	Ret (min)	ΔRI	MF	M82-C-T	M82-HS-T	Nag-C-T	Nag-HS-T	M82-C-Mi	M82-HS-Mi	Nag-C-Mi	Nag-HS-Mi
1_Ethanolamine	9.39	12.08	858	4832	6583	6265	4917	3818	6346	6364	5234
2_L-Valine	8.67	36.08	758	1779	1451	2400	2356	2024	1843	2349	2934
3_L-Proline	9.86	1.48	941	1389	2173	8150	7393	3694	1282	4273	6427
4_L-Serine	10.65	12.20	789	2460	1681	3051	2300	1863	1531	2072	2022
5_beta-Alanine	11.58	9.08	601	1249	412	1019	827	1194	807	1477	1076
6_L-Aspartic acid	12.72	1.76	862	6977	3873	8952	7275	4938	4020	6004	5920
7_L-Glutamic acid	13.92	9.00	812	2819	1725	4167	3794	2527	1972	3383	3446
8_L-Asparagine	14.51	11.52	903	9140	5339	9760	8919	15022	11389	9354	10495
9_L-Glutamine	15.68	3.57	608	9429	10749	12082	45266	14439	28740	12562	45227
10_Malic acid	12.32	2.42	943	2303	2370	2258	2066	1985	2303	2016	2574
11_Threonic acid	13.15	7.56	721	1026	923	751	1018	774	1273	1144	1205
12_2,3-Dihydroxybutanedioic acid	13.63	10.79	770	441	567	187	367	1864	797	1075	1316
13_Citric acid	16.10	0.80	940	18637	26795	19347	32677	17557	31428	20206	28624
14_D(-)-Quinic acid	16.47	2.34	875	1172	1791	1372	2564	1078	1433	1239	3120
15_Galactonic acid	17.84	6.61	866	1007	1761	1098	1495	886	1641	1184	1644
16_Glucaric acid	18.00	2.01	803	4360	996	1645	311	4096	1386	1873	381
17_Phosphoric acid	9.44	3.84	946	44665	32476	25562	25145	48168	51377	33383	30077
18_5-Aminocarboxy-4,6-dihydroxypyrimidine	12.19	5.71	678	2818	3949	3790	8497	2832	5086	3759	8421
19_3-cis-Caffèoylquinic acid	26.65	12.43	845	3331	2147	3371	2095	4814	2206	3291	1794
20_Metlhyfructofuranoside	15.61	2.11	587	106	*109*	*112*	*109*	255	*111*	344	129
21_Fructose	16.60	3.65	946	5675	4108	10702	15732	13962	5803	29857	23538
22_Glucose	16.86	8.21	946	1179	810	2395	3504	3041	1052	6571	4787
23_myo-Inositol	18.77	9.68	955	6893	8465	8666	12457	8117	8440	10298	13406
24_Sucrose	23.24	33.14	947	25362	32122	25964	53008	29036	43007	32460	48181
25_Sugars unknown	25.39	n.a.	n.a.	1025	631	594	675	798	689	683	587

Supplementary data table 1. *Cont.*

M82-C-P	M82-HS-P	Nag-C-P	M82-C-B	M82-HS-B	Nag-C-B	Nag-HS-B	M82-C-Ma	M82-HS-Ma	Nag-C-Ma	Nag-HS-Ma	ANOVA						
											C	G	D	CxG	CxD	GxD	CxGxD
5092	7036	5292	4577	5021	5673	5806	3211	6418	3084	4494							
2415	3481	2865	1524	1768	1488	3669	1645	1501	1316	1723	**X**	**X**	**X**	**X**	**X**		
17472	3149	12593	96282	13730	88447	15798	133517	76638	158879	178212	**X**	**X**	**X**		**X**	**X**	**X**
1656	2410	2090	243	418	198	1680	*109*	*113*	*107*	295			**X**		**X**		
738	1131	1433	*110*	209	*118*	667	*116*	*114*	*108*	*114*		**X**	**X**		**X**	**X**	
4175	3426	4595	4279	2462	2524	3559	2168	3621	1661	1932			**X**	**X**	**X**	**X**	**X**
2602	2314	3391	2068	1485	1975	2578	3316	2678	1730	1607		**X**	**X**	**X**		**X**	
14890	14816	5242	8812	14924	5035	7295	14185	20294	4836	11366		**X**	**X**		**X**	**X**	
17724	50293	9246	23849	61514	13245	39624	22760	91423	15198	59773	**X**		**X**	**X**	**X**	**X**	
3520	5364	2427	15556	18248	13519	6428	22587	39186	17385	41840	**X**	**X**	**X**		**X**	**X**	**X**
1070	2003	1144	874	2167	1001	2032	1110	1686	708	1495	**X**		**X**		**X**	**X**	**X**
2767	3648	2387	1043	2805	1363	2915	3414	4096	3718	3842	**X**		**X**		**X**	**X**	**X**
15787	21387	17681	13371	11465	9460	14385	63989	39551	43292	28840	**X**		**X**		**X**	**X**	
1282	1923	931	1367	6005	917	3598	7407	7935	7367	11806	**X**		**X**		**X**	**X**	
1022	1791	1124	679	1605	809	1524	1368	1965	1314	2612	**X**		**X**		**X**		**X**
3934	1090	1552	2930	1166	1404	216	7152	2066	1917	641	**X**	**X**	**X**				
48178	50814	30749	34203	39126	22274	31896	59950	72925	34397	55668		**X**	**X**		**X**		
2414	10333	2139	4205	4146	2454	7894	3016	9112	1081	7500	**X**				**X**		
4513	3193	3953	3174	2606	3003	2176	3632	4624	5391	5765	**X**		**X**		**X**	**X**	
1863	*114*	746	1531	724	1083	863	1097	725	490	765	**X**		**X**		**X**		
50618	26169	85038	51744	88958	122857	112825	48918	51336	119494	314124		**X**	**X**		**X**	**X**	**X**
8988	4693	16656	6182	14562	13051	15987	5897	6196	10351	26114		**X**	**X**		**X**		**X**
15136	11725	15743	4688	14791	14453	27152	6142	6659	10346	23390	**X**	**X**	**X**		**X**	**X**	**X**
32545	54336	41387	39508	33618	29901	45948	79641	57345	88144	106635			**X**		**X**		
593	471	492	160	266	135	267	*115*	*116*	*112*	*113*			**X**		**X**		

Supplementary data table 2. Average abundance of secondary metabolites per sample. Values in Italic light grey are random values below the detection threshold. Ret, retention time; ΔRt, delta retention time; Δppm, chemical shift; Ann. level., annotation level; Ref. reference. Cross in the ANOVA section represent metabolite that showed a statistical significant difference at the ANOVA analysis (p-value<0.001) for the factor condition (C), genotype (G), development (D), the interaction condition x genotype (CxG), the interaction condition x development (CxD), and the interaction condition x genotype x development (CxGxD); T, tetrad; Mi, Microspore; P, Polarized; B, Bicellular; Ma, Mature; Nag/N, Nagcarlang; C, control; HS/H, Heat stress; M, M82. 1, (Moco et al. 2006)) ; 2, (Iijima et al. 2008); 3, (Tikunov et al. 2010); 4, (Vallverdu-Queralt et al. 2010); 5, (Lawson et al. 1997); 6, (Noguchi et al. 2006); 7, (Roldan et al. 2014); 8, (van der Hooft et al. 2012); 9, (Strack et al. 1987); 10, chapter3 thesis; 11; https://metlin.scripps.edu/index.php

Name	Ret(Min)	Mass(D)	Δ Ret	Δ ppm	Ann. level	Ref.	M82-C-T	M82-HS-T	Nag-C-T	Nag-HS-T	M82-C-Mi	M82-HS-Mi	Nag-C-Mi
37_Hydroxytomatine I + FA	26.45	1094.5411	1.99	2.07	II	2	*8827*	3802221	2802488	8879929	2878366	5731280	5245753
38_Leptinidine trihexoside + FA	30.04	944.4894	2.21	3.52	IV	7,5,6	4616823	5515709	14194233	13611409	2774439	4375102	8308326
39_Acetoxy-tomatine + FA isomer I	30.32	1136.5522	n.a.	2.47	IV	7	1802262	1342351	2492030	1845420	4129786	2454217	4255887
40_Dehydrotomatine FA	30.06	1076.5308	0.11	2.28	III	7	2737865	3002894	4005028	7140909	4448789	3863413	5807040
41_Dehydrotomatine FA isomer I	29.42	1076.5308	0.75	2.28	III	7	3265209	3480669	5705780	6751403	4370883	3910327	5930083
42_Dehydrotomatine FA isomer II	28.83	1076.5308	1.34	2.28	III	7	1666006	2501812	3569177	6461081	3918162	3504057	5214950
43_alpha-Tomatin + FA	31.18	1078.5464	n.a.	2.25	IV	2	5534004	6300744	4941480	8507914	6829470	7113165	5838882
44_Hydroxytomatine + FA isomer I	22.40	1094.5417	n.a.	2.62	IV	7	941464	2024204	7083111	7054619	1667982	1599075	7457947
45_Hydroxytomatine + FA isomer II	23.73	1094.5416	n.a.	2.51	IV	7	1280568	1295182	8609565	7855517	2015705	1659341	8383505
46_Acetoxy-tomatine + FA isomer II	31.45	1136.5526	n.a.	2.79	IV	7	3177221	4739226	1474405	1410203	5721545	4388325	2685053
47_beta-tomatine + FA	31.03	946.5049	3.84	3.42	II	1	6385935	7240325	10994117	11148111	4738341	6401356	6808182
48_Hydroxytomatine + FA isomer III	21.24	1094.5410	n.a.	1.96	IV	7	488941	697877	8758186	8549556	1247877	945926	8496393
49_Hydroxytomatine isomer I	31.67	1048.5366	0.08	3.07	III	7	4019321	5962116	4803542	9937506	4974954	6382002	5100463
50_Hydroxytomatine + FA isomer IV	24.44	1094.5417	n.a.	2.62	IV	7	1322383	2779091	5779984	6780424	1861836	2212306	6365850
51_Acetoxy-tomatine isomer I	27.06	1090.5466	n.a.	2.45	IV	7	3868838	5968123	6804575	12443144	5162709	7045194	8027021
52_Glycoalkaloid +FA	33.31	1092.5619	n.a.	2.09	IV	2	3752619	7040745	4106596	7152183	5476206	7132790	4553014
53_Acetoxy-tomatine + FA isomer III	30.65	1136.5525	n.a.	2.68	IV	7	4987919	3078831	3161251	2239267	6914888	4487389	4541775
54_Acetoxy-tomatine isomer II	32.15	1090.5464	n.a.	2.22	IV	7	*9537*	3397295	3581787	8297132	2206789	3304155	6291044
55_beta-tomatine	30.18	900.4993	3.88	3.38	III	7	6685855	7349834	11672563	10482841	4277907	5995926	7391098
56_alpha-Tomatin	30.45	1032.5411	n.a.	2.57	IV	1	5188011	3986050	6078308	6236495	5753366	5406015	5285203
57_Hydroxynevirapine	25.51	281.1040	n.a.	1.52	IV	11	*9253*	*9210*	*8981*	*9264*	*9187*	*9269*	*9137*
58_Kaempferol 3,7-di-O-glucoside	21.70	609.1480	1.88	3.12	I	2	3110296	2427791	3808954	2714786	5353874	2642764	4177216
59_Eriodictyol	27.49	287.0566	n.a.	1.84	IV	7	*8699*	*10398*	*9317*	*8309*	*10875*	*8993*	*9864*

Supplementary data table 2. *Cont.*

Nag-HS-Mi	M82-C-P	M82-HS-P	Nag-C-P	M82-C-B	M82-HS-B	Nag-C-B	Nag-HS-B	M82-C-Ma	M82-HS-Ma	Nag-C-Ma	Nag-HS-Ma	ANOVA						
												C	G	D	CxG	CxD	GxD	CxGxD
9403345	3303133	8262312	5265964	4298091	7286719	6054458	7735712	6181859	8855717	11118525	10366171	X	X	X	X	X	X	X
9162868	2362713	3426638	7340158	229831	2023643	1495642	5964186	*9475*	*8705*	204433	*8395*		X	X		X	X	
2695273	7486967	6040431	7235006	9143405	6624229	9521950	6730909	13016438	10129053	12795875	11936701	X		X			X	
7834303	4977210	6013673	5817492	5987860	7153694	6886309	7965585	5736975	8133990	10564554	9021525	X	X	X		X	X	X
6600922	4802986	5180943	6452584	5483129	5625533	6451565	7154905	5956054	6342231	8527917	6999701		X	X			X	X
7876207	4852452	6362661	6603951	6273217	7625534	8783548	10681053	6429052	8342886	12496021	10654396	X	X	X		X	X	X
8548206	4756275	6009037	4342749	4670147	6330642	3544016	6207201	2983113	5362431	4003434	4486199	X		X			X	X
7919154	2536951	2672757	9894897	2715376	3154604	8745441	11157969	3103935	3475752	11928367	13880851		X	X		X	X	X
7139940	2787778	2414736	9938613	2308114	2058495	6398333	8002437	2213602	2033393	6706544	5609187	X	X	X		X	X	X
2191528	8029482	6644823	4549142	8661057	7442217	6865457	4550089	11730243	10690234	11446315	9622190		X	X		X	X	
7823264	4266191	5131075	6551978	1056370	2955276	1382092	5768889	*97077*	681209	204838	378152	X		X		X		
8140361	1912428	2017850	9402793	1971401	2216012	6586977	9255474	3048013	3237294	6960856	7709200		X	X		X	X	
10171111	3399314	5871672	4064557	4319654	6701708	4592612	8489655	3938939	6446987	5607216	7932820	X	X	X				X
7174293	2497799	2994153	8415671	2847728	3506935	8156640	10302296	4271179	4695831	10612775	10476314	X	X	X		X	X	
12526572	2698448	5074197	6407575	1957668	4114035	3411897	8399404	3282333	3380981	2514185	4986406							
7399934	5176528	8648020	4863110	4954158	8468328	3318162	6991221	6186236	9757194	5263823	7660808	X	X	X		X	X	
2732729	9315390	6678354	7523153	7858675	5355972	6733515	4835866	7484534	5379422	7581740	4912325	X	X	X			X	
8135213	4200752	7453086	5034755	5067431	10812014	4424294	7603672	6916243	11932952	6428268	8125812			X				
7919385	3829607	4138121	6791430	*9532*	471536	*9436*	4784100	*9965*	*9253*	*8662*	*8373*	X	X	X	X	X	X	X
5464371	5055732	5555026	5835536	6173203	5732845	5234226	6158359	5532806	5824340	7186956	5603153							
8517	*9165*	*9232*	*8896*	*9932*	*9521*	454888	*8844*	12854875	37042080	35227639	64466887			X				
3017507	5847320	5002774	4456376	10009549	7006568	7006702	4423051	12409107	10383932	11210563	8559824	X		X		X	X	X
190993	2254512	291678	2867636	12330603	24401106	15359303	8121835	20029049	32437234	19065539	35721178			X		X		

Supplementary data table 2. *Cont.*

Name	Ret(Min)	Mass(D)	Δ Ret	Δ ppm	Ann. level	Ref.	M82-C-T	M82-HS-T	Nag-C-T	Nag-HS-T	M82-C-Mi	M82-HS-Mi	Nag-C-Mi
60_Kaempferol 3-O-rutinoside	24.78	593.1523	n.a.	1.84	IV	1	2368980	1298517	2735869	1980613	4697654	2362805	4421312
61_Narginin-hexoside, -deoxyhexoside	22.25	579.1752	n.a.	5.70	IV	8	183844	9741461	*9937*	2383593	*8136*	6922317	305304
62_Kaempferol-hexoside-deoxyhexoside, -pentoside	21.96	725.1952	n.a.	2.40	IV	1,2	4866759	1948891	5098960	3967329	6584504	3333408	6367897
63_Quercetin-hexoside-deoxyhexoside, -pentoside	19.63	741.1905	n.a.	2.87	IV	1,2	7403924	3532098	8261620	6958658	8881922	4031037	7881121
64_Dihydrokaempferol-hexoside or Eriodictyol chalcone-hexoside 1	15.38	449.1106	n.a.	3.70	IV	2	*8479*	*9321*	*9680*	*10428*	*8075*	*10125*	*10088*
65_Quercetin 3-O-glucoside	22.72	463.0895	n.a.	2.83	IV	1,2	11863707	4066158	9278524	3412528	10934795	4048256	6990820
66_Dihydrokaempferol-hexoside or Eriodictyol chalcone-hexoside 2	14.07	449.1104	n.a.	3.22	IV	2	*8489*	*9585*	*9304*	*7212*	*8585*	*8784*	*9222*
67_Kaempferol 3,7-di-O-glucoside isomer I	20.06	609.1480	0.24	3.12	I	2	*9501*	*8782*	*9952*	*10253*	*10048*	*9744*	*9425*
68_Kaempferol-Hexoside	25.88	447.0940	2.43	1.52	III	7	4800224	1771105	6749228	3732178	5613018	3513236	7274029
69_Kaempferol 3-O-rutinoside-7-O-glucoside	15.03	755.2065	n.a.	3.34	IV	1,2	*9825*	276464	488369	1239930	*9895*	574169	2367823
70_Benzyl alcohol-hexoside-pentoside isomer I	10.83	401.1463	n.a.	2.52	IV	1,2	*8970*	*8978*	*7974*	*9138*	*9238*	*10302*	*9300*
71_Hexanol - pentoside - Hexoside + FA	22.50	441.1989	n.a.	2.57	IV	7	2335592	3223520	1582325	1982039	2088246	2651470	1540270
72_Methyl salicylate hexoside-pentoside + FA	16.84	491.1422	n.a.	3.10	IV	2,3	6287768	2146683	5946566	3645328	7395393	2715590	8286711
73_Guaiacol-hexoside-pentoside + FA	13.85	463.1471	n.a.	3.05	IV	3	6849138	6865106	4168075	7500789	9536959	8103336	5757673
74_Benzyl alcohol-hexoside-pentoside isomer II	13.64	401.1468	n.a.	3.58	IV	1,2	7759608	4844501	8618070	6205501	8491343	4934034	9187633
75_Coumaroylquinic acid	18.64	337.0937	1.70	2.37	II	1	6502025	5083172	7513813	5394191	5605821	5804151	8224494
76_Feruloylquinic acid-O-hexoside isomer I	13.31	529.1578	n.a.	2.83	IV	4	*8785*	*9798*	*9612*	*8784*	*8109*	*9323*	*8130*
77_5-Caffeoylquinic acid	14.81	353.0892	1.38	3.87	II	1,2	6490019	5966303	7561071	5256526	7337747	5256627	5670770
78_Ferulic acid hexoside isomer I	13.89	355.1047	n.a.	3.30	IV	1	2428854	2286898	2391313	1388402	683662	1220893	1787550
79_O-Feruloylquinic acid	20.20	367.1049	2.32	3.99	III	7	4685242	3780462	4488688	2616028	3652555	3373037	3286606
80_caffeoylglucaric isomer	6.54	371.0625	0.85	1.47	III	9	5237614	6692647	2755120	*8385*	14441687	6183654	5146991
81_Homovanillic acid-O-hexoside isomer I	11.05	343.1046	n.a.	3.29	IV	4	11595200	11590825	8651548	6177662	8231655	8727589	5333736
82_Homovanillic acid-O-hexoside isomer II	9.90	343.1047	n.a.	3.64	IV	4	8378086	5415810	6010615	4857499	8272231	6799907	4518152
83_5-Caffeoylquinic acid isomer I	11.82	353.0893	n.a.	4.21	IV	1	6054573	5102548	6471321	4800437	6925639	4959944	5934926
84_5-Caffeoylquinic acid isomer II	12.47	353.0890	n.a.	3.27	IV	1	6289064	8760157	7078450	4834819	8075357	6965354	4907243
85_caffeoylglucaric	7.69	371.0625	0.30	1.39	III	9	5559004	3837985	5308282	2218424	9965284	4003410	5748439
86_4-Hydroxycinnamyl alcohol 4-D-glucoside or 3-Hydroxychavicol 1-gluc	21.05	311.1147	n.a.	3.49	IV	11	897261	284873	2873002	1807078	*7721*	*9608*	4703245
87_cis-p-Coumaroylcorosolic acid-like	42.00	617.3925	n.a.	-13.47	IV	11	2171604	2150128	1308019	11873418	6245590	13925247	8794702
88_(R)-Rutaretin 1'-(6"-sinapoylglucoside)	5.84	629.1855	n.a.	3.26	IV	11	*9410*	*8663*	*8099*	*8213*	*9594*	*9248*	*9526*
89_Ferulic acid hexoside isomer II	10.47	355.1046	n.a.	3.26	IV	1	*9587*	374255	*9963*	179802	*10342*	*8499*	*59504*
90_O-Feruloylquinic acid isomer I	17.81	367.1049	n.a.	3.99	IV	7	4377816	2965570	3859669	2216472	3444562	2833917	3339237
91_Coumaroylquinic acid isomer	15.67	337.0937	n.a.	2.37	IV	1	4966874	2798268	4255811	3388348	6842903	3913694	8025152
92_Feruloylquinic acid-O-hexoside isomer II	9.07	529.1578	n.a.	2.94	IV	4	*9286*	*9714*	*8816*	*9399*	*10009*	*8809*	*10070*

Supplementary data table 2. *Cont.*

Nag-HS-Mi	M82-C-P	M82-HS-P	Nag-C-P	M82-C-B	M82-HS-B	Nag-C-B	Nag-HS-B	M82-C-Ma	M82-HS-Ma	Nag-C-Ma	Nag-HS-Ma	ANOVA						
												C	G	D	CxG	CxD	GxD	CxGxD
2523819	6147369	4990392	7271142	6450464	5995920	8327175	5052981	8492989	9102943	11009271	10943318	X	X	X		X	X	X
2336113	*9498*	894867	*9646*	509552	2786219	3210006	747508	23638341	29629412	30367489	34988026	X		X		X		X
4273904	7039752	4896780	8541890	6144397	4878534	7358878	5404158	5411463	4875478	7376902	4814067	X	X	X		X	X	X
6479744	5056187	3264517	4193094	5453968	3339949	3690229	4310779	5271560	2899181	4689896	2289058	X		X	X	X	X	X
64821	1884977	216222	647520	15111310	15865339	14497945	5559438	16462913	22707473	17281636	19409973			X		X	X	
3495614	5723498	4409559	6169699	3299477	3192067	4061355	4235624	2982986	2976970	5683861	4740144	X		X		X	X	
10447	*9689*	*7680*	*9730*	2256834	4150952	7758921	*10526*	26578471	26987642	21218430	30437259	X		X	X	X	X	X
9209	*8231*	*9227*	*8990*	21676191	4786515	25457337	*8278*	14255242	7004129	16245256	10300242	X	X	X	X	X	X	X
4003341	5454017	4182376	9691928	3165478	2998041	7393671	4872905	3285747	4398631	9307013	14620645	X	X	X		X	X	X
1832894	*9420*	1801276	2575262	1143132	3109237	5736417	2831503	16892152	20062496	33423028	43695471	X	X	X		X	X	
9221	*8259*	*8653*	*8074*	*8865*	*10268*	*8478*	*10052*	37118686	46342305	57450654	75132338			X			X	
1935499	1392380	1260418	414288	6771631	3561554	9125973	898281	20116929	12753154	36829189	22904002			X				
4218639	5771463	3223273	7888728	4078823	2490562	7031796	5054548	3514811	1851338	9088526	6718294	X	X	X	X	X	X	
9130441	10654344	9497072	7157136	6571583	6859829	6220635	11192156	1986333	2255155	3167138	5501254	X		X	X		X	
6151375	3390892	3751417	5700981	*8193*	2737835	4075325	3566376	4338467	6407424	6738982	7153089			X		X	X	X
5497465	5137923	3338625	9609804	1233825	1221768	5200448	5355407	2743988	5435477	5557973	7488084		X	X			X	
8409	*8359*	*7751*	*8782*	*9217*	*8399*	*8870*	*9607*	39995072	27710325	15383408	8409330			X		X	X	
4605308	6564410	5925258	6685772	6500525	4774237	6000530	6077514	5200022	5542392	6394615	6160460	X						
568363	*87893*	674112	228493	*9251*	238523	278694	168110	17760011	12020220	20305488	19081072			X		X		
2198321	3017202	2763194	3267937	2331945	2557527	3103059	2675106	12641822	13761617	14572578	12695897	X		X	X	X	X	
1508967	11961802	10248581	9283009	7744560	4998446	7199444	7751692	20672072	17304118	12130675	9232808	X	X	X			X	
4819914	6380902	7142100	3447839	4027504	6188363	1798854	3534963	3028009	4538259	1726837	1828628	X	X	X		X	X	X
3470795	8701165	7980491	4249394	8774810	7814942	3528331	3421373	6107445	5115793	3232224	1588685	X	X	X		X	X	X
4196007	6732754	5966041	6883956	6434668	4886552	6090484	5584379	6140207	6759179	7112644	7317550	X		X		X		
4470731	6985932	7398534	2552232	6275239	6100501	4497776	5763986	5433390	7977152	4761562	7575692							
2447414	8798306	6081205	8899108	7224229	3610636	6996958	4054949	7495352	5453772	8258914	4128021	X		X			X	
2096722	*8967*	*8571*	5331821	*8315*	*10114*	11613132	2967068	11885884	14258095	36067545	29746982		X	X			X	
11731117	1514609	2366096	2979579	*10718*	*9100*	*9578*	2586144	*10529*	*8517*	*9393*	*9796*	X	X	X	X			
9133	3884976	*8510*	*10101*	24529263	5823618	17632161	994843	33091178	26703021	22647105	32536454	X	X	X		X	X	
11045	*10009*	*9322*	*9699*	*8135*	*9590*	*7730*	*9550*	21592228	15461276	22903917	18106273			X		X		
1877248	2975227	2767137	2955295	2699968	2487817	3387175	2221207	14460552	16824976	17569784	16079337	X		X	X	X	X	
4261027	7386240	4767646	13439444	2077560	1387463	5369293	5467008	2770755	5447703	4296656	5277042	X	X	X		X	X	X
9936	*9498*	*9941*	*9869*	*9833*	*9868*	*10144*	*8640*	51496519	34888699	18452948	9266895		X	X		X	X	

Supplementary data table 2. *Cont.*

Name	Ret(Min)	Mass(D)	Δ Ret	Δ ppm	Ann. level	Ref.	M82-C-T	M82-HS-T	Nag-C-T	Nag-HS-T	M82-C-Mi	M82-HS-Mi	Nag-C-Mi
93_Tricoumaroyl spermidine isomer I	37.47	582.2626	n.a.	-2.77	II	10	*69290*	668332	350739	594855	6063034	2747017	2914389
94_Tricoumaroyl spermidine isomer II	38.49	582.2625	n.a.	-2.56	II	10	322263	792884	392659	496944	5534228	1822181	3512513
95_Feruloyl dicoumaroyl spermidine isomer I	38.89	612.2732	n.a.	-2.77	II	10	186222	1251247	317184	277398	6376284	2535781	3274087
96_Dicoumaroylspermidine isomer I	16.77	436.2254	n.a.	-2.83	IV	11	5251857	2143616	976874	2048650	7399360	5171960	3266114
97_Caffeoyl dicoumaroyl spermidine isomer I	36.07	598.2574	n.a.	-2.63	II	10	*8997*	399386	*9565*	*107796*	6641444	1400677	3604777
98_Dicoumaroylspermidine isomer II	18.37	436.2255	n.a.	-2.90	IV	11	5262247	2796050	1799719	2187680	8505209	3888058	4375160
99_Feruloyl dicoumaroyl spermidine isomer II	39.52	612.2732	n.a.	-2.77	II	10	175138	1109551	378096	157060	6299308	2314931	3055293
100_Tricoumaroyl spermidine isomer III	39.76	582.2625	n.a.	-2.66	II	10	1666174	1035284	1098495	1149056	6020963	2288063	10323023
101_Tricoumaroyl spermidine isomer IV	39.22	582.2626	n.a.	-2.87	II	10	320873	633763	289314	319797	5798431	1388043	3804839
102_Caffeoyl dicoumaroyl spermidine isomer II	36.70	598.2575	n.a.	-2.74	II	10	*8617*	293463	*9770*	*9893*	7053332	1072181	3713687
103_Tricoumaroyl spermidine isomer V	40.26	582.2632	n.a.	-3.92	II	10	*9212*	527668	*8703*	*9563*	5851358	756753	1074438
104_Dicoumaroylspermidine isomer III	17.56	436.2254	n.a.	-2.83	IV	11	2044512	941896	682054	1276045	8325547	3424199	4895201
105_Dicoumaroylputrescine	32.24	379.1675	n.a.	-3.19	IV	11	*9031*	*10730*	*9220*	*10588*	6631850	*9513*	2372729
106_Dicoumaroylspermidine IV	19.07	436.2256	n.a.	-3.11	IV	11	4265328	2174900	1380459	1671351	8560552	2935385	4540451
107_Caffeoyl dicoumaroyl spermidine isomer III	37.20	598.2575	n.a.	-2.74	II	10	167702	520671	160454	186601	6898320	1066208	3755764
108_Feruloyl dicoumaroyl spermidine isomer III	41.05	612.2732	n.a.	-2.77	II	10	283425	953334	*8661*	*9917*	7301467	845595	2736291
109_Caffeoyl dicoumaroyl spermidine isomer IV	37.69	598.2574	n.a.	-2.63	II	10	*7643*	*9640*	*8921*	*9170*	6962118	642880	3217497
110_Diferuloylspermidine	20.76	496.2461	n.a.	-1.60	IV	11	5557575	7152809	1107993	2245452	10982883	10726262	3840170

Supplementary data table 2. *Cont.*

Nag-HS-Mi	M82-C-P	M82-HS-P	Nag-C-P	M82-C-B	M82-HS-B	Nag-C-B	Nag-HS-B	M82-C-Ma	M82-HS-Ma	Nag-C-Ma	Nag-HS-Ma	ANOVA					
												C	G	D	CxG	CxD	GxD	CxGxD
4741086	7125741	8074074	6403169	10680192	6175387	6805869	11625494	3116452	2441353	2215625	2273316			**X**				
3822742	8283968	7761939	8344366	7621722	6221083	7212026	10747931	3406943	3392684	2800770	2929103			**X**				
3194871	9843937	9856919	7582998	8851080	7817818	6916489	12052682	4164440	3402165	2592074	3557143			**X**				
6614590	6106045	6619708	10532454	3794530	2973836	5753508	11303382	1865834	1932261	3031551	2545460			**X**			**X**	
4473917	10209175	11450858	9479966	6496967	9168613	6656749	13532598	4654267	5242986	3756990	4049729			**X**				
5346243	6805018	4195302	12200966	3125799	1971893	5802375	8617991	1903358	1284615	3851212	2257610		**X**	**X**	**X**		**X**	**X**
2960223	11498853	13129875	8093118	8224656	8637726	6987410	11408410	6043535	6398533	3792403	4129868			**X**				
5740289	11339401	9932082	11063284	6137377	6583593	7487821	10928622	6161448	5031106	7405749	4449670			**X**				
3365783	8582243	8848661	8754145	6309872	6128827	7006220	9361464	4753869	4970760	4745907	4400610			**X**				
4204815	11270154	12642358	10315532	6096045	9858666	5936619	13483588	4747418	5564913	3810834	4123989			**X**		**X**		**X**
904542	6825502	6219441	6777111	6483360	5660020	6147054	6915154	4734216	5856636	4918931	5866031			**X**				
6006438	10594440	9390756	18286059	3770454	4083360	7115703	12727116	1923037	1999141	3994546	4150267			**X**			**X**	
8845	25476450	25689504	19826535	11106009	15014436	8042855	7921828	5306307	7098477	2958938	*7633*	**X**	**X**	**X**		**X**	**X**	**X**
4145725	7988447	4868462	12085553	3195663	2184480	5417962	6735919	3107905	2533505	5855014	4259098	**X**		**X**	**X**		**X**	**X**
3353111	11398300	10145363	10079437	5991586	9024945	6329135	10138719	6674951	6968518	5860900	6639570			**X**				
817877	13745420	10238187	10448723	7097363	8264270	7042531	9443613	7980221	7762470	5850501	7201307		**X**	**X**		**X**	**X**	
2746192	13579062	11476101	10831777	6114636	9975210	5819297	9367728	8083641	7861616	5967698	7558637			**X**			**X**	
7474790	10417400	9912533	5398868	1857053	6076732	*9284*	5980609	2687545	4976511	1250707	4975316			**X**				

Supplementary data table 3. Single abundance , average and fold change of primary metabolites and secondary metabolites per sample. Values in Italic light grey are random values below the detection threshold. The fold change represent the division of the value A by a value B (fold change = A/B), when the value A is higher than B, the fold change is positive and if not negative. C, control; HS, heat stress; T, tetrad; Mi, microspore; P, polarized; B, bicellular; Ma, Mature and Nag, nagcarlang

Compound	Fold change															
	T/Mi	T/P	T/B	T/Ma	Mi/P	Mi/B	Mi/Ma	P/B	P/Ma	B/Ma	C/HS	M82/Nag	M82C/M82HS	NagC/NagHS	M82C/NagC	M82HS/NagHS
1_Ethanolamine	1.03	-1.04	1.06	1.30	-1.07	1.03	1.26	1.10	1.35	1.22	-1.19	1.01	-1.46	1.04	-1.24	1.23
2_L-Valine	-1.13	-1.44	-1.04	1.31	-1.28	1.08	1.48	1.38	1.89	1.37	-1.17	-1.20	-1.08	-1.28	-1.11	-1.31
3_L-Proline	1.25	-2.25	-10.90	**-27.85**	-2.82	-13.67	-34.91	-4.84	-12.36	-2.55	1.52	-1.50	2.51	1.05	-1.08	-2.58
4_L-Serine	1.29	1.17	3.80	15.44	-1.10	2.95	12.00	3.23	13.15	4.07	1.01	-1.24	1.05	-1.05	-1.19	-1.30
5_beta-Alanine	-1.26	-1.22	3.26	7.98	1.03	4.12	10.08	3.98	9.74	2.45	1.26	-1.24	1.26	1.24	-1.22	-1.24
6_L-Aspartic acid	1.33	1.70	2.16	2.95	1.28	1.63	2.23	1.27	1.73	1.37	1.15	-1.18	1.30	1.02	-1.05	-1.35
7_L-Glutamic acid	1.13	1.16	1.58	1.37	1.02	1.40	1.21	1.37	1.19	-1.15	1.16	-1.23	1.30	1.03	-1.10	-1.39
8_L-Asparagine	-1.37	-1.38	-1.07	-1.50	-1.01	1.28	-1.10	1.29	-1.09	-1.41	-1.22	1.62	-1.10	-1.39	1.81	1.44
9_L-Glutamine	-1.27	-1.30	-1.74	-2.38	-1.02	-1.37	-1.87	-1.34	-1.84	-1.37	**-3.25**	1.20	-2.84	-3.81	1.42	1.06
10_Malic acid	1.01	-1.68	**-5.99**	-13.49	-1.70	-6.05	-13.63	-3.56	-8.02	-2.25	-1.63	1.15	-1.52	-1.76	1.22	1.06
11_Threonic acid	-1.18	-1.51	-1.63	-1.34	-1.28	-1.38	-1.14	-1.08	1.12	1.21	-1.61	1.11	-1.69	-1.51	1.02	1.14
12_2,3-Dihydroxybutanedioic acid	-3.31	-7.69	-5.33	-9.88	-2.32	-1.61	-2.98	1.44	-1.28	-1.85	-1.26	1.14	-1.29	-1.21	1.09	1.17
13_Citric acid	-1.01	1.33	1.99	-1.81	1.34	2.01	-1.80	1.50	-2.40	-3.61	-1.09	1.09	-1.01	-1.19	1.18	-1.00
14_D(-)-Quinic acid	1.00	1.25	-1.73	-5.01	1.25	-1.73	-5.02	-2.16	-6.26	-2.90	-1.87	-1.15	-1.59	-2.23	1.04	-1.35
15_Galactonic acid	-1.02	1.00	1.14	-1.38	1.02	1.16	-1.36	1.14	-1.38	-1.57	-1.70	-1.04	-1.77	-1.64	-1.11	-1.04
16_Glucaric acid	-1.03	-1.17	1.31	-1.57	-1.13	1.35	-1.52	1.53	-1.34	-2.06	**3.37**	2.68	3.32	4.33	2.68	3.50
17_Phosphoric acid	-1.28	-1.35	1.00	-1.75	-1.06	1.28	-1.37	1.36	-1.29	-1.75	-1.14	1.51	-1.06	-1.22	1.61	1.40
18_5-Aminocarboxy-4,6-dihydroxypy	-1.05	-1.03	1.03	-1.08	1.01	1.07	-1.03	1.06	-1.04	-1.11	-2.56	-1.05	-2.17	-3.05	1.16	-1.22
19_3-cis-Caffeoylquinic acid	-1.09	-1.40	1.01	-1.75	-1.28	1.10	-1.60	1.42	-1.25	-1.77	1.29	1.01	1.30	1.29	1.02	1.01
20_Metlhyfructofuranoside	-1.92	-8.32	-9.63	-7.05	-4.33	-5.01	-3.67	-1.16	1.18	1.37	1.85	1.31	2.65	1.19	1.75	-1.27
21_Fructose	-1.96	-5.79	**-10.10**	-14.33	-2.95	-5.14	-7.30	-1.74	-2.47	-1.42	-1.35	-2.62	-1.07	-1.58	**-2.15**	**-3.19**
22_Glucose	-1.90	-4.97	-6.12	-5.97	-2.62	-3.22	-3.14	-1.23	-1.20	1.03	-1.19	-2.06	-1.12	-1.28	**-1.94**	**-2.23**
23_myo-Inositol	-1.10	-1.55	-1.67	-1.27	-1.41	-1.52	-1.16	-1.08	1.22	1.31	-1.41	-1.66	-1.23	-1.60	-1.45	-1.89
24_Sucrose	-1.12	-1.25	-1.09	-2.42	-1.12	1.02	-2.17	1.15	-1.94	-2.23	-1.25	-1.22	-1.08	-1.46	-1.06	-1.42
25_Sugars unknown	1.07	1.42	3.56	6.45	1.33	3.33	6.03	2.51	4.54	1.81	1.12	1.19	1.26	-1.02	1.33	1.04
37_Hydroxytomatine I + FA	-1.50	-1.45	-1.64	-2.36	1.04	-1.09	-1.57	-1.13	-1.63	-1.44	-1.66	-1.47	-2.04	-1.49	-1.83	-1.34
38_Leptinidine trihexoside + FA	1.54	2.17	3.91	164.23	1.41	2.53	106.58	1.80	75.78	42.05	-1.18	-2.64	-1.54	-1.14	-3.16	-2.34
39_Acetoxy-tomatine + FA isomer I	-1.81	-3.70	-4.28	-6.40	-2.05	-2.37	-3.54	-1.16	-1.73	-1.50	1.30	-1.06	1.34	1.25	-1.02	-1.09
40_Dehydrotomatine FA	-1.30	-1.33	-1.66	-1.98	-1.02	-1.28	-1.52	-1.25	-1.49	-1.20	-1.17	-1.39	-1.18	-1.21	-1.38	-1.42
41_Dehydrotomatine FA isomer I	-1.08	-1.14	-1.29	-1.45	-1.05	-1.19	-1.34	-1.13	-1.27	-1.13	-1.02	-1.39	-1.03	-1.04	-1.38	-1.40
42_Dehydrotomatine FA isomer II	-1.44	-1.67	-2.35	-2.67	-1.16	-1.63	-1.85	-1.40	-1.60	-1.14	-1.19	-1.56	-1.22	-1.22	-1.58	-1.57
43_alpha-Tomatin + FA	-1.12	1.26	1.22	1.50	1.41	1.37	1.68	-1.03	1.20	1.23	-1.38	-1.00	-1.26	-1.53	1.09	-1.11
44_Hydroxytomatine + FA isomer I	-1.09	-1.18	-1.51	-1.89	-1.08	-1.38	-1.74	-1.28	-1.61	-1.26	-1.05	-3.96	-1.18	-1.11	-4.11	-3.87

Supplementary data table 3. *Cont.*

45_Hydroxytomatine + FA isomer II	-1.01	-1.06	1.01	1.15	-1.05	1.02	1.16	1.08	1.22	1.13	1.20	-3.80	1.12	1.12	-3.77	-3.78
46_Acetoxy-tomatine + FA isomer II	-1.39	-2.37	-2.55	-4.03	-1.71	-1.84	-2.90	-1.07	-1.70	-1.58	1.12	1.43	1.10	1.22	1.38	1.53
47_beta-tomatine + FA	1.39	1.68	3.20	26.28	1.21	2.31	18.93	1.91	15.62	8.20	-1.24	-1.46	-1.35	-1.21	-1.57	-1.40
48_Hydroxytomatine + FA isomer III	-1.02	1.04	-1.08	-1.13	1.06	-1.06	-1.11	-1.13	-1.18	-1.05	1.03	-4.61	-1.05	-1.05	-4.64	-4.62
49_Hydroxytomatine isomer I	-1.08	1.39	1.03	1.03	1.50	1.10	1.11	-1.36	-1.35	1.01	-1.68	-1.30	-1.52	-1.89	-1.17	-1.46
50_Hydroxytomatine + FA isomer IV	-1.06	-1.11	-1.49	-1.80	-1.05	-1.41	-1.71	-1.34	-1.62	-1.21	-1.09	-2.84	-1.26	-1.10	-3.07	-2.68
51_Acetoxy-tomatine isomer I	-1.13	1.54	1.63	2.05	1.73	1.83	2.31	1.06	1.33	1.26	-1.61	-1.71	-1.51	-1.76	-1.60	-1.87
52_Glycoalkaloid +FA	-1.11	-1.13	-1.08	-1.31	-1.01	1.03	-1.18	1.05	-1.16	-1.22	-1.64	1.17	-1.61	-1.65	1.16	1.12
53_Acetoxy-tomatine + FA isomer III	-1.39	-2.33	-1.84	-1.88	-1.68	-1.33	-1.36	1.27	1.24	-1.02	1.50	1.25	1.46	1.61	1.24	1.36
54_Acetoxy-tomatine isomer II	-1.30	-1.46	-1.83	-2.19	-1.12	-1.40	-1.68	-1.25	-1.50	-1.20	-1.74	-1.16	-2.01	-1.56	-1.40	-1.09
55_beta-tomatine	1.41	1.84	6.86	998.28	1.30	4.85	705.71	3.73	542.81	145.49	-1.12	-1.66	-1.21	-1.12	-1.75	-1.61
56_alpha-Tomatin	-1.02	-1.02	-1.08	-1.12	-1.00	-1.06	-1.10	-1.06	-1.10	-1.04	1.03	-1.09	1.05	1.01	-1.07	-1.11
57_Hydroxynevirapine	1.02	1.01	-13.16	-4075.13	-1.01	-13.38	-4142.59	-13.28	-4110.56	-309.59	-2.32	-2.23	-2.88	-2.26	-2.77	-2.17
58_Kaempferol 3,7-di-O-glucoside	-1.26	-1.69	-2.36	**-3.53**	-1.34	-1.87	-2.80	-1.39	-2.09	-1.50	1.31	1.17	1.34	1.31	1.20	1.17
59_Eriodictyol	-6.01	-196.57	-1639.66	-2920.61	-32.70	-272.80	-485.91	-8.34	-14.86	-1.78	-1.56	1.02	-1.65	-1.48	-1.08	1.04
60_Kaempferol 3-O-rutinoside	-1.67	-2.93	-3.08	-4.72	-1.75	-1.84	-2.82	-1.05	-1.61	-1.53	1.26	-1.16	1.19	1.32	-1.20	-1.08
61_Narginin-hexoside, -deoxyhexosi	1.29	10.11	1.70	-9.63	7.85	1.32	-12.39	-5.95	-97.34	-16.35	-1.72	-1.11	-2.05	-1.49	-1.39	-1.01
62_Kaempferol-hexoside-deoxyhexc	-1.29	-1.72	-1.50	-1.42	-1.33	-1.16	-1.09	1.15	1.21	1.06	1.52	-1.18	1.51	1.51	-1.16	-1.16
63_Quercetin-hexoside-deoxyhexosi	-1.04	1.57	1.56	1.73	1.63	1.62	1.80	-1.01	1.10	1.11	1.47	-1.10	**1.88**	1.15	1.12	-1.47
64_Dihydrokaempferol-hexoside or E	-2.46	-96.68	-1346.24	-2001.18	-39.36	-548.11	-814.76	-13.92	-20.70	-1.49	-1.08	1.13	-1.16	1.04	1.03	1.24
65_Quercetin 3-O-glucoside	1.12	1.32	1.94	1.75	1.17	1.72	1.55	1.47	1.33	-1.11	1.74	1.00	1.86	1.62	1.08	-1.06
66_Dihydrokaempferol-hexoside or E	-1.07	-1.04	-409.87	-3041.99	1.03	-382.78	-2840.98	-392.38	-2912.21	-7.42	-1.18	-1.10	-1.08	-1.31	-1.00	-1.22
67_Kaempferol 3,7-di-O-glucoside isc	1.00	1.09	-1349.23	-1242.09	1.09	-1351.36	-1244.06	-1472.57	-1355.64	1.09	3.16	-1.21	3.04	3.23	-1.16	-1.09
68_Kaempferol-Hexoside	-1.20	-1.51	-1.08	-1.85	-1.26	1.11	-1.55	1.40	-1.23	-1.72	1.28	-1.92	1.32	1.19	-1.81	-2.02
69_Kaempferol 3-O-rutinoside-7-O-g	-2.38	-2.90	-6.36	-56.62	-1.22	-2.68	-23.84	-2.19	-19.51	-8.90	-1.34	-2.38	-1.43	-1.39	-2.47	-2.40
70_Benzyl alcohol-hexoside-pentosic	-1.09	1.05	-1.07	-6162.12	1.14	1.01	-5676.23	-1.13	-6484.95	-5736.12	-1.43	-1.76	-1.25	-1.63	-1.55	-2.03
71_Hexanol - pentoside - Hexoside +	1.11	2.23	-2.23	-10.15	2.01	-2.48	-11.27	-4.98	-22.64	-4.55	1.45	-1.53	1.39	1.43	-1.51	-1.48
72_Methyl salicylate hexoside-pento	-1.25	-1.25	-1.03	-1.17	1.00	1.21	1.07	1.21	1.06	-1.13	1.83	-1.63	2.18	1.56	-1.41	-1.98
73_Guaiacol-hexoside-pentoside + FA	-1.28	-1.43	-1.22	1.97	-1.12	1.05	2.52	1.18	2.82	2.39	-1.20	1.04	1.06	-1.57	1.34	-1.24
74_Benzyl alcohol-hexoside-pentosic	-1.05	1.60	2.64	1.11	1.68	2.77	1.17	1.65	-1.44	-2.37	1.15	-1.37	1.06	1.19	-1.43	-1.27
75_Coumaroylquinic acid	-1.03	1.02	1.88	1.15	1.04	1.93	1.18	1.85	1.14	-1.63	1.16	-1.58	1.02	1.22	-1.70	-1.42
76_Feruloylquinic acid-O-hexoside is	1.09	1.11	1.02	-2474.29	1.02	-1.06	-2693.45	-1.09	-2756.83	-2535.09	1.38	2.56	1.44	1.46	2.60	2.63
77_5-Caffeoylquinic acid	1.11	-1.01	1.08	1.08	-1.12	-1.02	-1.02	1.09	1.10	1.00	1.17	-1.02	1.17	1.17	-1.01	-1.01
78_Ferulic acid hexoside isomer I	1.99	6.43	12.23	-8.14	3.23	6.13	-16.23	1.90	-52.37	-99.58	1.10	-1.37	1.28	-1.06	-1.19	-1.61
79_O-Feruloylquinic acid	1.24	1.29	1.46	-3.45	1.04	1.17	-4.29	1.13	-4.45	-5.03	1.07	-1.03	1.00	1.14	-1.09	1.04
80_caffeoylglucaric isomer	-1.86	-2.86	-1.88	-4.04	-1.54	-1.02	-2.18	1.52	-1.41	-2.14	1.36	1.73	1.32	1.58	1.64	1.96
81_Homovanillic acid-O-hexoside iso	1.40	1.68	2.44	3.42	1.20	1.74	2.44	1.46	2.03	1.40	-1.12	1.72	-1.15	1.02	1.59	1.87
82_Homovanillic acid-O-hexoside iso	1.07	-1.13	1.05	1.54	-1.21	-1.02	1.44	1.19	1.74	1.47	1.20	1.89	1.21	1.29	1.87	1.99

Supplementary data table 3. *Cont.*

83_5-Caffeoylquinic acid isomer I	1.02	-1.16	-1.03	-1.22	-1.19	-1.04	-1.24	1.14	-1.05	-1.19	1.18	-1.01	1.17	1.19	-1.01	1.01
84_5-Caffeoylquinic acid isomer II	1.10	1.19	1.19	1.05	1.08	1.08	-1.05	-1.00	-1.14	-1.14	-1.17	1.36	-1.13	-1.19	1.39	1.31
85_caffeoylglucaric	-1.31	-1.87	-1.29	-1.50	-1.43	1.01	-1.14	1.45	1.25	-1.16	1.86	1.16	1.70	2.19	1.11	1.43
86_4-Hydroxycinnamyl alcohol 4-D-gl	-1.16	-1.22	-2.49	-15.69	-1.05	-2.14	-13.49	-2.05	-12.89	-6.30	1.29	-3.94	-1.14	1.32	-4.73	-3.14
87_cis-p-Coumaroylcorosolic acid-lik	-2.33	1.91	6.69	457.78	4.45	15.56	1064.39	3.50	239.23	68.41	-2.15	-1.54	-1.85	-2.50	-1.32	-1.77
88_(R)-Rutaretin 1'-(6''-sinapoylgluco	-1.09	-151.37	-1424.47	-3343.87	-138.79	-1306.07	-3065.93	-9.41	-22.09	-2.35	1.39	1.15	1.89	-1.04	1.53	-1.29
89_Ferulic acid hexoside isomer II	6.42	14.82	16.39	-136.09	2.31	2.55	-873.29	1.11	-2016.80	-2230.10	1.18	-1.22	1.36	1.00	-1.06	-1.44
90_O-Feruloylquinic acid isomer I	1.17	1.16	1.24	-4.84	-1.01	1.06	-5.65	1.07	-5.60	-6.01	1.06	-1.06	1.00	1.11	-1.11	-1.00
91_Coumaroylquinic acid isomer	-1.50	-2.21	1.08	-1.15	-1.48	1.61	1.30	2.39	1.92	-1.24	1.46	-1.41	1.31	1.54	-1.47	-1.26
92_Feruloylquinic acid-O-hexoside is	-1.04	-1.05	-1.03	-3066.12	-1.01	1.01	-2939.08	1.02	-2920.04	-2964.88	1.43	2.80	1.48	1.59	2.79	3.01
93_Tricoumaroyl spermidine isomer I	-9.78	-17.11	-20.96	-5.97	-1.75	-2.14	1.64	-1.23	2.87	3.51	1.05	1.12	1.35	-1.29	1.45	-1.20
94_Tricoumaroyl spermidine isomer I	-7.33	-16.22	-15.86	-6.25	-2.21	-2.16	1.17	1.02	2.60	2.54	1.12	1.01	1.26	-1.01	1.13	-1.13
95_Feruloyl dicoumaroyl spermidine	-7.57	-17.90	-17.54	-6.75	-2.37	-2.32	1.12	1.02	2.65	2.60	1.03	1.23	1.18	-1.15	1.42	1.04
96_Dicoumaroylspermidine isomer I	-2.15	-2.98	-2.29	1.11	-1.38	-1.06	2.39	1.30	3.31	2.54	1.04	-1.18	1.30	-1.19	1.04	-1.49
97_Caffeoyl dicoumaroyl spermidine	-30.66	-78.97	-68.20	-33.67	-2.58	-2.22	-1.10	1.16	2.35	2.03	-1.07	1.10	1.01	-1.18	1.19	-1.00
98_Dicoumaroylspermidine isomer II	-1.84	-2.57	-1.62	1.30	-1.40	1.13	2.38	1.58	3.33	2.10	1.48	-1.30	1.81	1.22	-1.09	-1.63
99_Feruloyl dicoumaroyl spermidine	-8.04	-23.97	-19.37	-11.19	-2.98	-2.41	-1.39	1.24	2.14	1.73	-1.02	1.40	1.02	-1.05	1.45	1.35
100_Tricoumaroyl spermidine isomer	-4.92	-8.71	-6.29	-4.66	-1.77	-1.28	1.06	1.38	1.87	1.35	1.31	-1.18	1.26	1.34	-1.19	-1.12
101_Tricoumaroyl spermidine isomer	-9.18	-22.33	-18.42	-12.07	-2.43	-2.01	-1.31	1.21	1.85	1.53	1.15	1.02	1.17	1.13	1.05	1.01
102_Caffeoyl dicoumaroyl spermidin	-49.87	-141.84	-109.95	-56.71	-2.84	-2.20	-1.14	1.29	2.50	1.94	-1.08	1.16	-1.01	-1.15	1.23	1.08
103_Tricoumaroyl spermidine isomer	-15.47	-47.61	-45.40	-38.50	-3.08	-2.94	-2.49	1.05	1.24	1.18	1.18	1.18	1.26	1.11	1.26	1.11
104_Dicoumaroylspermidine isomer	-4.58	-10.32	-5.60	-2.44	-2.25	-1.22	1.88	1.84	4.23	2.30	1.26	-1.41	1.34	1.16	-1.31	-1.52
105_Dicoumaroylputrescine	-228.03	-2392.22	-1063.60	-388.47	-10.49	-4.66	-1.70	2.25	6.16	2.74	1.32	**2.11**	1.01	3.34	1.46	4.81
106_Dicoumaroylspermidine IV	-2.13	-3.50	-1.85	-1.66	-1.65	1.15	1.28	1.90	2.11	1.11	1.61	-1.22	1.85	1.39	-1.08	-1.43
107_Caffeoyl dicoumaroyl spermidin	-14.56	-40.72	-30.41	-25.25	-2.80	-2.09	-1.73	1.34	1.61	1.20	1.07	1.14	1.12	1.03	1.19	1.09
108_Feruloyl dicoumaroyl spermidin	-9.32	-36.57	-25.37	-22.94	-3.92	-2.72	-2.46	1.44	1.59	1.11	1.24	1.33	1.30	1.19	1.40	1.28
109_Caffeoyl dicoumaroyl spermidin	-383.58	-1352.67	-884.18	-833.14	-3.53	-2.31	-2.17	1.53	1.62	1.06	1.10	1.28	1.16	1.05	1.34	1.22
110_Diferuloylspermidine	-2.06	-2.14	1.15	1.16	-1.04	2.37	2.38	2.46	2.47	1.00	-1.53	1.96	-1.23	-2.23	2.71	1.50

Supplementary data table 4. Average abundance of metabolites per samples that showed a significant statistical difference at the three way interaction condition x genotype x development. Values in Italic light grey are random values below the detection threshold. In the fold change section values that are highlighted in grey showed a significant statistical difference for the respective fold change ratio (p-value < 0.05 at the Bonferroni post hoc test). Values in white showed more than 2-fold change ; T, tetrad; Mi, Microspore; P, Polarized; B, Bicellular; Ma, Mature; Nag, Nagcarlang; C, control; HS, Heat stress

Name	M82HS / M82C fold change					NagHS / NagC fold change				M82 C / Nag C fold change					M82 HS / Nag HS fold change			
	T	Mi	P	B	Ma	T	Mi	B	Ma	T	Mi	P	B	Ma	T	Mi	B	Ma
3_L-Proline	1.56	-2.88	-5.55	-7.01	-1.74	-1.10	1.50	-5.60	1.12	-5.87	-1.16	1.39	1.09	-1.19	-3.40	-5.01	-1.15	-2.33
6_L-Aspartic acid	-1.80	-1.23	-1.22	-1.74	1.67	-1.23	-1.01	1.41	1.16	-1.28	-1.22	-1.10	1.70	1.30	-1.88	-1.47	-1.45	1.87
10_Malic acid	1.03	1.16	1.52	1.17	1.73	-1.09	1.28	-2.10	2.41	1.02	-1.02	1.45	1.15	1.30	1.15	-1.12	2.84	-1.07
11_Threonic acid	-1.11	1.64	1.87	2.48	1.52	1.36	1.05	2.03	2.11	1.37	-1.48	-1.07	-1.15	1.57	-1.10	1.06	1.07	1.13
12_2,3-Dihydroxybutanedioic acid	1.29	-2.34	1.32	2.69	1.20	1.96	1.22	2.14	1.03	2.35	1.73	1.16	-1.31	-1.09	1.54	-1.65	-1.04	1.07
15_Galactonic acid	1.75	1.85	1.75	2.36	1.44	1.36	1.39	1.88	1.99	-1.09	-1.34	-1.10	-1.19	1.04	1.18	-1.00	1.05	-1.33
21_Fructose	-1.38	-2.41	-1.93	1.72	1.05	1.47	-1.27	-1.09	2.63	-1.89	-2.14	-1.68	-2.37	-2.44	-3.83	-4.06	-1.27	-6.12
22_Glucose	-1.46	-2.89	-1.92	2.36	1.05	1.46	-1.37	1.22	2.52	-2.03	-2.16	-1.85	-2.11	-1.76	-4.33	-4.55	-1.10	-4.21
23_myo-Inositol	1.23	1.04	-1.29	3.15	1.08	1.44	1.30	1.88	2.26	-1.26	-1.27	-1.04	-3.08	-1.68	-1.47	-1.59	-1.84	-3.51
37_Hydroxytomatine I + FA	430.73	1.99	2.50	1.70	1.43	3.17	1.79	1.28	-1.07	-317.48	-1.82	-1.59	-1.41	-1.80	-2.34	-1.64	-1.06	-1.17
40_Dehydrotomatine FA	1.10	-1.15	1.21	1.19	1.42	1.78	1.35	1.16	-1.17	-1.46	-1.31	-1.17	-1.15	-1.84	-2.38	-2.03	-1.11	-1.11
41_Dehydrotomatine FA isomer I	1.07	-1.12	1.08	1.03	1.06	1.18	1.11	1.11	-1.22	-1.75	-1.36	-1.34	-1.18	-1.43	-1.94	-1.69	-1.27	-1.10
42_Dehydrotomatine FA isomer II	1.50	-1.12	1.31	1.22	1.30	1.81	1.51	1.22	-1.17	-2.14	-1.33	-1.36	-1.40	-1.94	-2.58	-2.25	-1.40	-1.28
43_alpha-Tomatin + FA	1.14	1.04	1.26	1.36	1.80	1.72	1.46	1.75	1.12	1.12	1.17	1.10	1.32	-1.34	-1.35	-1.20	1.02	1.20
44_Hydroxytomatine + FA isomer I	2.15	-1.04	1.05	1.16	1.12	-1.00	1.06	1.28	1.16	-7.52	-4.47	-3.90	-3.22	-3.84	-3.49	-4.95	-3.54	-3.99
45_Hydroxytomatine + FA isomer II	1.01	-1.21	-1.15	-1.12	-1.09	-1.10	-1.17	1.25	-1.20	-6.72	-4.16	-3.57	-2.77	-3.03	-6.07	-4.30	-3.89	-2.76
49_Hydroxytomatine isomer I	1.48	1.28	1.73	1.55	1.64	2.07	1.99	1.85	1.41	-1.20	-1.03	-1.20	-1.06	-1.42	-1.67	-1.59	-1.27	-1.23
55_beta-tomatine	1.10	1.40	1.08	49.47	-1.08	-1.11	1.07	507.02	-1.03	-1.75	-1.73	-1.77	1.01	1.15	-1.43	-1.32	-10.15	1.11
58_Kaempferol 3,7-di-O-glucoside	-1.28	-2.03	-1.17	-1.43	-1.20	-1.40	-1.38	-1.58	-1.31	-1.22	1.28	1.31	1.43	1.11	-1.12	-1.14	1.58	1.21
60_Kaempferol 3-O-rutinoside	-1.82	-1.99	-1.23	-1.08	1.07	-1.38	-1.75	-1.65	-1.01	-1.15	1.06	-1.18	-1.29	-1.30	-1.53	-1.07	1.19	-1.20
61_Narginin-hexoside, -deoxyhexoside	52.99	850.78	94.22	5.47	1.25	239.88	7.65	-4.29	1.15	18.50	-37.52	-1.02	-6.30	-1.28	4.09	2.96	3.73	-1.18
62_Kaempferol-hexoside-deoxyhexoside, -pentoside	-2.50	-1.98	-1.44	-1.26	-1.11	-1.29	-1.49	-1.36	-1.53	-1.05	1.03	-1.21	-1.20	-1.36	-2.04	-1.28	-1.11	1.01
63_Quercetin-hexoside-deoxyhexoside, -pentoside	-2.10	-2.20	-1.55	-1.63	-1.82	-1.19	-1.22	1.17	-2.05	-1.12	1.13	1.21	1.48	1.12	-1.97	-1.61	-1.29	1.27
66_Dihydrokaempferol-hexoside or Eriodictyol chalcone-hexoside 2	1.13	1.02	-1.26	1.84	1.02	-1.29	1.13	-737.15	1.43	-1.10	-1.07	-1.00	-3.44	1.25	1.33	-1.19	394.37	-1.13
67_Kaempferol 3,7-di-O-glucoside isomer I	-1.08	-1.03	1.12	-4.53	-2.04	1.03	-1.02	-3075.15	-1.58	-1.05	1.07	-1.09	-1.17	-1.14	-1.17	1.06	578.19	-1.47
68_Kaempferol-Hexoside	-2.71	-1.60	-1.30	-1.06	1.34	-1.81	-1.82	-1.52	1.57	-1.41	-1.30	-1.78	-2.34	-2.83	-2.11	-1.14	-1.63	-3.32
74_Benzyl alcohol-hexoside-pentoside isomer II	-1.60	-1.72	1.11	334.15	1.48	-1.39	-1.49	-1.14	1.06	-1.11	-1.08	-1.68	-497.39	-1.55	-1.28	-1.25	-1.30	-1.12
81_Homovanillic acid-O-hexoside isomer I	-1.00	1.06	1.12	1.54	1.50	-1.40	-1.11	1.97	1.06	1.34	1.54	1.85	2.24	1.75	1.88	1.81	1.75	2.48
82_Homovanillic acid-O-hexoside isomer II	-1.55	-1.22	-1.09	-1.12	-1.19	-1.24	-1.30	-1.03	-2.03	1.39	1.83	2.05	2.49	1.89	1.11	1.96	2.28	3.22
91_Coumaroylquinic acid isomer	-1.77	-1.75	-1.55	-1.50	1.97	-1.26	-1.88	1.02	1.23	1.17	-1.17	-1.82	-2.58	-1.55	-1.21	-1.09	-3.94	1.03
98_Dicoumaroylspermidine isomer II	-1.88	-2.19	-1.62	-1.59	-1.48	1.22	1.22	1.49	-1.71	2.92	1.94	-1.79	-1.86	-2.02	1.28	-1.38	-4.37	-1.76
102_Caffeoyl dicoumaroyl spermidine isomer II	34.06	-6.58	1.12	1.62	1.17	1.01	1.13	2.27	1.08	-1.13	1.90	1.09	1.03	1.25	29.66	-3.92	-1.37	1.35
105_Dicoumaroylputrescine	1.19	-697.16	1.01	1.35	1.34	1.15	-268.26	-1.02	-387.65	-1.02	2.80	1.28	1.38	1.79	1.01	1.08	1.90	929.97
106_Dicoumaroylspermidine IV	-1.96	-2.92	-1.64	-1.46	-1.23	1.21	-1.10	1.24	-1.37	3.09	1.89	-1.51	-1.70	-1.88	1.30	-1.41	-3.08	-1.68

Supplementary data table 5. Average abundance of metabolites per genotype and condition that showed a significant statistical difference at the two way interaction condition x genotype. In the fold change section values that are highlighted in grey showed a significant statistical difference for the respective fold change ratio (p-value < 0.05 at the Bonferroni post hoc test). Values in white showed more than 2-fold change; C, control; HS, Heat stress; Nag, Nagcarlang

Name	M82C	M82HS	NagC	NagHS	M82HS/M82C	NagHS/NagC	M82C/NagC	M82HS/NagHS
2_L-Valine	1878	2032	2084	2670	1.08	1.28	-1.11	-1.31
7_L-Glutamic acid	2666	2048	2929	2856	-1.30	-1.03	-1.10	-1.39
9_L-Glutamine	17640	50119	12467	47473	**2.84**	**3.81**	1.42	1.06
72_Methyl salicylate hexoside-pentoside + F	5409652	2485489	7648465	4909202	**-2.18**	-1.56	-1.41	-1.98
73_Guaiacol-hexoside-pentoside + FA	7119671	6716100	5294131	8331160	-1.06	1.57	1.34	-1.24
79_O-Feruloylquinic acid	5265753	5247167	5743774	5046338	-1.00	-1.14	-1.09	1.04
87_cis-p-Coumaroylcorosolic acid-like	1990610	3691818	2620254	6550119	1.85	**2.50**	-1.32	-1.77
90_O-Feruloylquinic acid isomer I	5591625	5575883	6222232	5598566	-1.00	-1.11	-1.11	-1.00

Table 6. Average abundance of metabolites per genotype and development that showed a significant statistical difference at the two way interaction development x genotype. Values in Italic light grey are random values below the detection threshold. In the fold change section values that are highlighted in grey showed a significant statistical difference for the respective fold change ratio (p-value < 0.05 at the Bonferroni post hoc test). Values in white showed more than 2-fold change. T, tetrad; Mi, Microspore; P, Polarized; B, Bicellular; Ma, Mature; Nag, Nagcarlang; C, control; HS, Heat stress

Name	M82-T	M82-Mi	M82-P	M82-B	M82-Ma	Nag-T	Nag-Mi	Nag-P	Nag-B	Nag-Ma	M82/Nag fold change T	Mi	B	Ma
5_beta-Alanine	877	1001	934	160	115	923	1277	1433	393	111	-1.05	-1.28	-2.46	1.04
7_L-Glutamic acid	2333	2250	2458	1776	2997	3980	3415	3391	2277	1669	-1.71	-1.52	-1.28	1.80
8_L-Asparagine	7451	13206	14853	11868	17240	9340	9924	5242	6165	8101	-1.25	1.33	1.93	2.13
9_L-Glutamine	10015	21590	34008	42682	57092	28674	28895	9246	26434	37486	-2.86	-1.34	1.61	1.52
13_Citric acid	22262	24493	18587	12418	51770	26012	24415	17681	11922	36066	-1.17	1.00	1.04	1.44
14_D(-)-Quinic acid	1447	1255	1603	3686	7671	1968	2179	931	2258	9587	-1.36	-1.74	1.63	1.25
19_3-cis-Caffeoylquinic acid	2805	3510	3853	2890	4128	2733	2543	3953	2590	5578	1.03	1.38	1.12	1.35
38_Leptinidine trihexoside + FA	5066266	3574770	2894675	1126737	*9090*	13902821	8735597	7340158	3729914	106414	-2.74	-2.44	-3.31	11.71
39_Acetoxy-tomatine + FA isomer I	1572306	3292002	6763699	7883817	11572746	2168725	3475580	7235006	8126430	12366288	-1.38	-1.06	-1.03	1.07
46_Acetoxy-tomatine + FA isomer II	3958224	5054935	7337153	8051637	11210238	1442304	2438291	4549142	5707773	10534253	2.74	2.07	1.41	1.06
48_Hydroxytomatine + FA isomer III	593409	1096902	1965139	2093706	3142654	8653871	8318377	9402793	7921225	7335028	-14.58	-7.58	-3.78	2.33
50_Hydroxytomatine + FA isomer IV	2050737	2037071	2745976	3177332	4483505	6280204	6770071	8415671	9229468	10544544	-3.06	-3.32	-2.90	2.35
52_Glycoalkaloid +FA	5396682	6304498	6912274	6711243	7971715	5629390	5976474	4863110	5154692	6462316	-1.04	1.05	1.30	1.23
53_Acetoxy-tomatine + FA isomer III	4033375	5701138	7996872	6607323	6431978	2700259	3637252	7523153	5784691	6247033	1.49	1.57	1.14	1.03
64_Dihydrokaempferol-hexoside or Eriodictyol chalcone-hexoside 1	*8900*	*9100*	1050599	15488325	19585193	*10054*	37455	647520	10028692	18345805	-1.13	-4.12	1.54	1.07
65_Quercetin 3-O-glucoside	7964932	7491526	5066528	3245772	2979978	6345526	5243217	6169699	4148490	5212003	1.26	1.43	-1.28	1.75
69_Kaempferol 3-O-rutinoside-7-O-glucoside	143144	292032	905348	2126185	18477324	864150	2100359	2575262	4283960	38559250	-6.04	-7.19	-2.01	2.09
70_Benzyl alcohol-hexoside-pentoside isomer I	*8974*	*9770*	*8456*	*9567*	41730495	*8556*	*9260*	*8074*	*9265*	66291496	1.05	1.06	1.03	1.59
72_Methyl salicylate hexoside-pentoside + FA	4217225	5055491	4497368	3284693	2683074	4795947	6252675	7888728	6043172	7903410	-1.14	-1.24	-1.84	2.95
73_Guaiacol-hexoside-pentoside + FA	6857122	8820148	10075708	6715706	2120744	5834432	7444057	7157136	8706396	4334196	1.18	1.18	-1.30	2.04
75_Coumaroylquinic acid	5792599	5704986	4238274	1227796	4089733	6454002	6860980	9609804	5277928	6523029	-1.11	-1.20	-4.30	1.59
76_Feruloylquinic acid-O-hexoside isomer I	*9292*	*8716*	*8055*	*8808*	33852698	*9198*	*8269*	*8782*	*9238*	11896369	1.01	1.05	-1.05	2.85
79_O-Feruloylquinic acid	4232852	3512796	2890198	2444736	13201720	3552358	2742463	3267937	2889083	13634237	1.19	1.28	-1.18	1.03
80_caffeoylglucaric isomer	5965131	10312671	11105191	6371503	18988095	1381753	3327979	9283009	7475568	10681741	4.32	3.10	-1.17	1.78
85_caffeoylglucaric	4698495	6984347	7439756	5417433	6474562	3763353	4097926	8899108	5525954	6193467	1.25	1.70	-1.02	1.05
86_4-Hydroxycinnamyl alcohol 4-D-glucoside or 3-Hydroxychavicol 1-gl	591067	*8664*	*8769*	*9215*	13071990	2340040	3399984	5331821	7290100	32907264	-3.96	-392.41	-791.16	2.52
88_(R)-Rutaretin 1'-(6''-sinapoylglucoside)	*9037*	*9421*	1946743	15176440	29897099	*8156*	*9330*	*10101*	9313502	27591780	1.11	1.01	1.63	1.08
90_O-Feruloylquinic acid isomer I	3671693	3139240	2871182	2593892	15642764	3038071	2608242	2955295	2804191	16824560	1.21	1.20	-1.08	1.08
92_Feruloylquinic acid-O-hexoside isomer II	*9500*	*9409*	*9719*	*9851*	43192609	*9108*	*10003*	*9869*	*9392*	13859922	1.04	-1.06	1.05	3.12
96_Dicoumaroylspermidine isomer I	3697737	6285660	6362877	3384183	1899048	1512762	4940352	10532454	8528445	2788506	2.44	1.27	-2.52	1.47
104_Dicoumaroylspermidine isomer III	1493204	5874873	9992598	3926907	1961089	979049	5450820	18286059	9921410	4072406	1.53	1.08	-2.53	2.08
108_Feruloyl dicoumaroyl spermidine isomer III	618380	4073531	11991804	7680817	7871346	*9289*	1777084	10448723	8243072	6525904	66.57	2.29	-1.07	1.21
109_Caffeoyl dicoumaroyl spermidine isomer IV	*8641*	3802499	12527582	8044923	7972628	*9046*	2981844	10831777	7593512	6763168	-1.05	1.28	1.06	1.18

Chapter 7

General discussion

Pollen quality is a determining factor for the production of fruit. The sensitivity of pollen fertility to high temperatures has been associated with alterations of metabolite profiles (Chapter 2). Since most of the studies published previously were carried out using targeted analytical approaches we hypothesised that the use of untargeted metabolomics approaches would broaden the identification of metabolites that play a role in the heat stress response of male reproductive organs, and of metabolites that are associated with thermo-tolerance. To achieve this goal, we first assessed the suitability of the common pollen isolation procedure for different metabolomics platforms and used them to identify metabolites that are associated with thermo-tolerance. In the following part of this thesis I discuss different aspects of the knowledge that we gained on the topic and how this might serve future research in this and in related research fields.

Metabolomics and pollen, how far can we go?

The analyses of metabolites, as well as that of proteins and transcripts, in developing pollen requires the isolation of pollen which is tightly enclosed within the anthers. To achieve this, the pollen is isolated in a solution (Honys and Twell 2004, Chaturvedi et al. 2013, Firon et al. 2006). In chapter two, we showed that such an isolation method applied to tomato pollen not only led to contamination with metabolic content of the anther mother tissue, but also to rehydration of the pollen grains, which impairs the metabolic steady-state of the mature pollen. We did not find any literature addressing how the common way to isolate pollen, which requires pollen incubation in a solution, affects the metabolic composition of this specific plant tissue. The purity of a pollen sample is a major concern when using this method, which is supported by our results showing metabolite contamination, for instance, with alkaloids. The assessment of cross contamination upon isolation of specific tissues with metabolites from surrounding tissues remains difficult due to analytical limitations. Different research groups determined the abundance of sugars and the amino acid proline in different fractions of the male reproductive organs, including (i) pollen grains, (ii) locular fluid and (iii) the anther walls (Pressman et al. 2012, Castro and Clement 2007, Mutters et al. 1989). Although precautions were taken to prevent cross contamination of the samples isolated from adjacent tissues (e.g. filtration, washing, microscopic observations), our data suggest that the purity of such preparations might have been overestimated. Also, the current method used to isolate pollen grains needs to be considered as suboptimal, especially in studies of pollen development, when morphology of the pollen and the supportive tissues undergo changes that may have an effect on the efficiency of the isolation procedures. Therefore, greater attention should be payed to the development of methods that prevent isolation drawbacks such as the above-mentioned rehydration and cross contamination.

Another issue, yet to be addressed, is on the study of metabolic changes that occur in response to high temperature using samples with different proportions of viable and non-viable pollen (viability ratios). Analysis of sugar content has already been performed on pollen that showed different pollen viability due to exposure to high temperatures (Firon et al. 2006). Non-viable pollen are qualified as empty cells that do not contain cytoplasm (Iwahori 1965). Therefore, a lower abundance of metabolites in samples with lower pollen viability might then be largely caused by a higher number of empty cells in these samples. This effect can mask the metabolic

changes that take place in response to high temperature the fraction of intact pollen. We found that the amount of sugars positively correlated with a high viability of pollen samples created artificially by pooling viable and dead pollen in different proportions (data not-shown).

This issue raises the question – how to assess the relative quantitative contribution of morphological (e.g. empty pollen) and physiological (e.g. stress response) effects when studying metabolic processes in pollen subjected to heat stress?

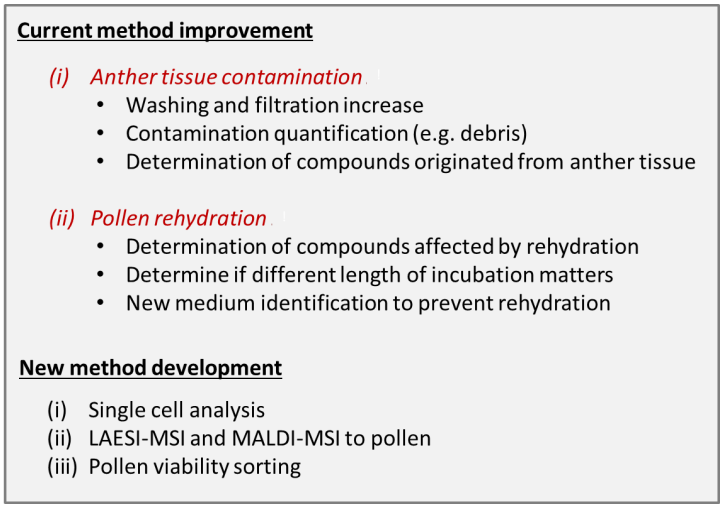


Figure 1. Toward an optimal method to analyse pollen

Metabolomics approaches are growing technologies. In the last decade the use of metabolomic platforms have broadened their applications towards the localisation of metabolites within plant organs or within cells (Etalo et al. 2015). Platforms such as MALDI-MSI (Matrix-Assisted Laser Desorption/Ionization – Mass Spectrometry Imaging) (Cornett et al. 2007) and LAESI-MSI (Laser Ablation Electrospray Ionization - Mass Spectrometry Imaging) (Etalo et al. 2015) might be the future for the analysis of reproductive organs. Pioneer experiments were performed in flower buds of *Arabidopsis thaliana*, to identify the spatial localization of glucosinolates (Sarsby et al. 2012). A similar approach within the different fractions of the anthers, including the anther wall, tapetum cells, and pollen grains might allow following the repartition of metabolites during pollen development or in response to heat stress. It might even be possible to target specifically the metabolome of pollen at a normal temperature condition compared to pollen that are exposed to different lengths and strengths of temperature stresses. However, the lack of resolution of current analytical platforms and the complex preparation of pollen samples may still be limiting factors. The resolution of LAESI-MSI, which can be used to analyse fresh plant material, is not sufficient yet to analyse the metabolic profile within the single pollen grains, whereas the MALDI-MSI, which has a resolution of 5-9 µm (Korte et al. 2015), might be more suitable for tomato pollen of 20µm size, although sample preparation is required in this case and must be adapted to minimize its impact on the metabolic composition.

To date, the most reliable method to analyse the pollen metabolome remains the analysis of dry mature pollen that is not altered by any sample preparation, and therefore represents its real biological state. If other, less optimal, methods are used (e.g. solution incubation) the possible drawbacks and limitations should be mentioned and taken into account carefully when interpreting such results. We summarized the different concerns of the pollen isolation and gave suggestions for further improvement toward an optimized method for the use of metabolomics in pollen research (Figure 1).

Metabolites associated with tolerance of reproductive tissue, what have we learnt?

To determine metabolites responsive to high temperatures and possibly associated with tolerance we started our investigation on the pollen cell (Chapter 3), the quality of which is directly linked to fruit production. We observed that short heat stress did not induce a strong metabolic change in the pollen grain, while we had evidence that it did in the whole anther (data not shown). Anthers are the main tissues that support pollen during development and we hypothesized that the metabolic changes in response to heat stress occur in anthers first and then affect the metabolic composition of pollen. In Chapter 5 we performed metabolic profiling of the whole anther of thermo-tolerant and thermo-sensitive tomato varieties in order to identify metabolites associated with pollen thermo-tolerance.

In Chapter 5, we identified specific metabolites that might be involved in the tolerance of tomato cultivar Nagcarlang, such as sugars, proline, specific flavonoids and polyamines and alkaloids. Our data suggest that the microspore stage of the pollen development is the most sensitive to mild chronic high temperatures, which could be either due to degeneration of the tapetal cells that might alter the pollen nutrition or to lower energy availability under heat stress that might lead to starvation. Final proof for a causal-effect relationship needs to be further addressed; as it might be that the metabolome alteration is the consequence of upstream events (e.g. early tapetum degeneration) rather than the cause of fertility loss. Although, as I mentioned above, we did not observe a strong metabolic response in pollen of another thermo-sensitive tomato variety MicroTom upon a short heat stress; a total pool of flavonoids tended to increase (Chapter 3). This contrast with the observations of Chapter 5 might indicate several points that will require further investigation: genotypic variation, different response to different stress durations or even relocation of some metabolites between different generative tissues.

Nevertheless, the use of untargeted metabolomics approaches allowed us to identify new metabolic candidates such as alkaloids, and specifically flavonoids and polyamines that might play a role in the protection from oxidative stress. Hence, taken all together, the results from Chapter 3 and Chapter 5 strongly indicate that a well-running antioxidant system and the presence of osmolytes are essential to overcome high temperatures and that a higher basal level of sugars might provide better resources to face high energy demands.

From exploratory to targeted analyses

Metabolomics analysis is a powerful tool that allows the detection of hundreds of metabolites. While the detection of metabolites involved in a stress response is relatively easy, their identification requires far greater efforts. We have identified new metabolites associated with the heat tolerance of Nagcarlang, including flavonoids, polyamines and alkaloids. The secondary plant metabolome is extremely diverse and metabolite annotation strongly depends on the availability of mass spectra information in related databases. One of the critical points that influences the informativeness of the metabolomics data derived using LC-MS is the annotation precision. In our study of generative tomato organs we annotated the LC-MS-derived metabolites by comparing the experimental fragmentation and retention characteristics of metabolites analysed in pollen and anthers with an in-house database, which was developed in a large part based on metabolic profiling of tomato fruit (Moco et al. 2006). Hence, more investigations should be carried out to confirm the annotation of the specific metabolites associated with thermo-tolerance and, especially, to discover the structure of many unknown compounds correlated with pollen viability to ensure a proper understanding of the metabolic response under high temperatures.

Untargeted metabolomics approaches aim to detect as many as possible metabolites in a given extract. However, the initial step of any metabolomic profiling experiment - the extraction procedure - might not be optimal for all the compounds in the extract. This may limit the detection and realistic quantification of specific metabolites. We have identified several metabolites associated with thermo-tolerance. The use of adapted extraction protocols for specific classes of metabolites (e.g. flavonoids) might allow the detection of a larger number of metabolites involved in the related pathways. Such an approach could pin point the metabolic steps which are critical for the different levels of metabolites found at different environmental conditions.

Production of tomato even under global warming conditions

In view of maintaining a sustainable tomato production under hot conditions as a result of climate change, every strategy is worth to be exploited in order to develop or to find tomato genotypes tolerant to high temperatures. In Chapter 2 of this thesis we proposed a strategy to use metabolic markers in order to develop tomatoes that are more tolerant to high temperatures (Paupière et al. 2014). We have identified several metabolites that might be used as markers due to their association with the tolerance such as glucose and fructose, the amino acid proline, flavonoids and polyamines. In our study, we only used two genotypes, hence we cannot exclude that the metabolic profile was specific to these two genotypes tested at the specific conditions used, rather than to be attributed to a general tolerance response. Therefore, further validation of the robustness of the metabolic markers is required. Some associations can however be supported by other independent studies. Considering the hexose sugars, several independent studies showed that impairment in their metabolism cause a failure in pollen development (Pressman et al. 2002, Firon et al. 2006, Li et al. 2015) supporting the idea that these metabolites are good candidate metabolic markers for thermo-tolerance. Regarding the other groups of metabolites, such as the alkaloids, specific polyamines and flavonoids, further assessments need

to be done, using a wider panel of genetic variation and different conditions (e.g. genotypes from Chapter 4). Segregating populations (e.g. cross between M82 and Nagcarlang) are another type of material which can be used for genetic analysis to confirm the ability of certain metabolic processes to provide better tolerance under high temperatures.

In Chapter 4, we have screened the pollen viability of different genotypes to high temperatures. This led to the identification of M82 and Nagcarlang as genotypes with contrasting heat tolerance. In our screening, we have only assessed the pollen viability since it is known to be highly correlated with fruit production (Kartikeya et al. 2012). However, to ensure the production of tomatoes under changing environments, one of the main concerns of a breeder is the actual production of tomato fruit. As mentioned in Chapter 4, the tolerance to high temperatures is positively correlated with small fruit size, indicating a possible linkage between these two traits (Wessel-Beaver and Scott 1992). In line with that we observed that fruits of M82 (sensitive genotype) were bigger than the ones of Nagcarlang (tolerant genotype). Performing a genetic analysis on offspring plants from a cross between Nagcarlang and M82 might allow the identification of chromosomal regions linked with the heat stress tolerance. In case the genetic loci responsible for thermo-tolerance and fruit size are genetically linked it will be very difficult to separate the fruit size and thermo-tolerance trait. This remains however, to be determined.

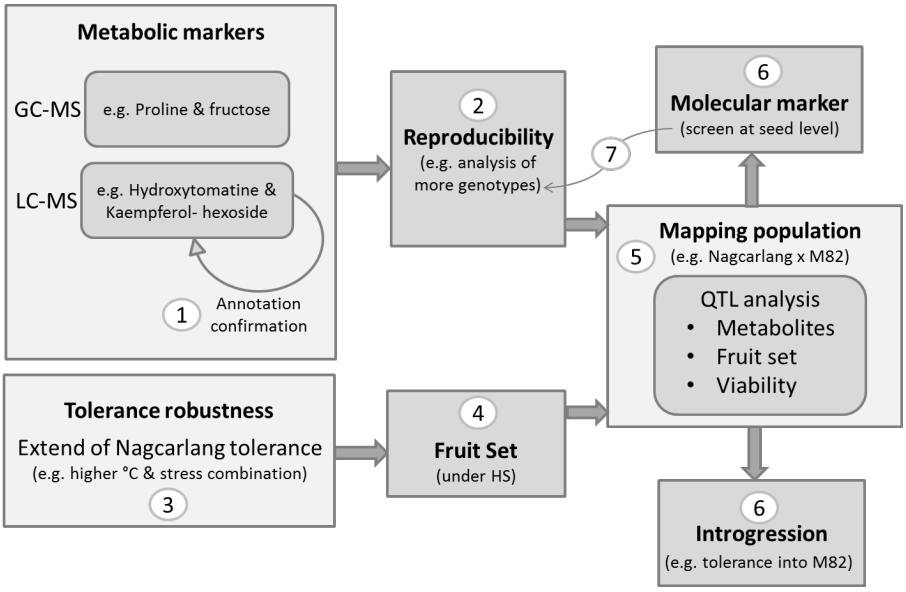


Figure 2. Work flow for a breeding approach for temperature tolerant tomatoes using different marker types. °C, temperature; HS, heat stress; QTL, quantitative trait locus.

A schematic overview of the various steps needed to breed for thermo-tolerance using metabolic markers is shown in Figure 2. We identified putative metabolites associated with

thermo-tolerance that can be integrated into a breeding program. At first, the annotation of metabolites detected by LC-MS needs to be confirmed, due to their poor annotation level (1). Then the reproducibility of these metabolites as metabolic markers for tolerance should be assessed in other genotypes to confirm the association with thermo-tolerance (2). Before these metabolic markers can be used to screen a mapping population derived from a cross between Nagcarlang and M82 for thermo-tolerance, the robustness of the thermo-tolerance of Nagcarlang should be determined under more extreme stresses (e.g. longer and stronger heat stresses) in order to ensure a reliable use of these metabolic markers (3). Although pollen viability strongly correlates with fruit set, it is pivotal to ensure production of seeded fruit of Nagcarlang under heat stress before investing into a mapping population (4). Once the above-mentioned requirements are met, a mapping population can be developed, phenotyped with metabolic markers and genotypes with molecular markers, in order to identify QTLs and molecular markers linked with thermo-tolerance (5). The identified molecular markers can then be used to screen for plant tolerance at the seedling level (6). The association of these genetic loci linked with thermo-tolerance can be further screened with their traits of interest (e.g. pollen viability and fruit production) in a broader panel of known thermo-tolerant genotypes to assess their use in other germplasms (7). At last, chromosomal regions associated with thermo-tolerance found in the mapping population can be introgressed by marker-assisted backcrossing into M82 in order to improve the tolerance of the M82 genotype.

Our study was carried out under highly controlled environmental conditions (climate chamber) while a large part of the world's tomato production is done in the field, especially for processing tomatoes and partly for fresh tomatoes. The tolerance to high temperatures is a complex trait that is influenced by different factors which makes high temperature tolerance a very difficult trait to breed for (Chapter 2). In view of climate change, the heat waves do not come alone but are highly associated with drought, and the combination of these two stresses leads to more severe effects than the individual stresses alone (Lipiec et al. 2013). Hence, the exploration of stress combinations seems to be essential to ensure the tomato production under global warming conditions. This topic should be addressed in follow-up research. For instance, to what extent is the genotype Nagcarlang able to tolerate high temperatures? We have only assessed mild chronic heat stress for two weeks, but the stability of Nagcarlang under more variable climate conditions with stronger heat waves or in combination with another stress, such as drought, remains to be explored.

SPOT-ITN converges to a similar result: Microsporogenesis is an important cellular process

As previously mentioned in the general introduction, our project is part of a European Consortium (http://spot-itn.eu/) that aimed to improve the understanding of tomato pollen thermo-tolerance by investigating different levels of organization including transcripts, proteins and metabolites. As described above our metabolomic data strongly suggested that the microsporogenesis is a pivotal step of the pollen development to ensure fertility of the mature pollen grain. Our collaboration with the partners of Frankfurt University (Germany) who are focused on the mechanistic response of Heat Shock Factors (HSFs) and Heat Shock Proteins (HSPs) led to similar conclusions. The exposure of pollen to short heat stress of antisense

HSFA2 tomato plants (cv. Moneymaker) led to a reduction of pollen viability only when the stress was applied during meiosis and microsporogenesis (Fragkostefanakis et al. 2016). The results demonstrated that these young developmental stages had a lower ability to induce a proper heat stress response (HSR). Besides, the metabolic study of these young anthers showed that the antisense *HSFA2* tomato plants accumulated lower levels of specific sugars compared with the non-transgenic control, both under control conditions and after a short heat stress (Fragkostefanakis et al. 2016). When comparing one thermo-sensitive and one thermo-tolerant genotype, we found a similar relationship between sugar abundance and temperature tolerance supporting the hypothesis that lower accumulation of energy resources might lead to a higher sensitivity to high temperatures. Interestingly, other partners from Radboud University (The Netherlands) overexpressed *HSFA2* in tomato plants (cv. MicroTom) under control of a tapetum cell-specific promoter. This led to a higher expression of HSFA2 at microspore stage and a better pollen viability of the transgenic plants compared to the control line when exposed to high temperatures (Li 2015). Hence, improving the tolerance of the microspore stage is a valuable strategy to improve pollen quality under adverse environmental conditions. In parallel, Vienna University (Austria) and Volcani Centre Agricultural Research Organization (Israel) performed a proteome analysis of post-meiotic pollen (combination of microspore and polarized pollen cells) and mature pollen stages of tomato (cv. Hazera 3017) under control and short heat stress conditions. These experiments also led to the conclusion that microspore and polarized pollen stages are crucial in the heat stress responses (Chaturvedi et al. 2015). At this specific stage, they found accumulation of proteins linked with the ROS scavenger ascorbate peroxidase which is in line with the strong accumulation of flavonoid antioxidants we found in the polarized pollen stage of the cv. MicroTom after short heat stress. Although all these studies were performed in different tomato genotypes they all converged into the same direction, indicating that microsporogenesis is pivotal for pollen fertility under high temperatures and that targeting this stage for improvement can lead to a better pollen performance under high temperatures.

Why is pollen so sensitive to high temperatures?

Many studies focused on elucidating the factors leading to a decrease of pollen viability under high temperatures. Our approach showed that the loss of tomato pollen fertility under mild chronic heat stress is associated with alterations in metabolite accumulation during microsporogenesis. This provides new leads, in addition to the range of already existing factors that might contribute to the sensitivity of pollen to high temperatures. However, one question always remains: why the pollen grain, the carrier of precious genetic material and therefore an important contributor of the survival of species is the most sensitive organ to high temperatures? Recently, Muller and Rieu (2016) suggested what causes the failure of developing a mature and fertile pollen grain under high temperatures including (i) a nutrition failure, a reason that we can support with the results from our study in Chapter 5, in which we observed a drop of hexoses in the microspore stage anthers of the sensitive genotype and (ii) a less efficient heat stress response (HSR), since several heat shock proteins do not accumulate as much in pollen as they do in leaves under heat stress. In general, I can think of three major reasons why the pollen viability of tomato is so sensitive to high temperatures: (i) Domestication - Tomato originated

from South America where a range of diverse climatic variation exists, including extreme warm temperatures (Bergougnoux 2014). Over time, humans have domesticated tomatoes by focusing on traits of interest such as yield, shape, and pest resistance (Bergougnoux 2014). Such extensive breeding has narrowed the genetic variation of the cultivated tomato compared to wild tomato species (Viquez-Zamora et al. 2013). The lower diversity of domesticated tomatoes might have led to a loss of temperature tolerance. This hypothesis is supported by the observation that wild relatives are in general more tolerant to abiotic stresses than domesticated crops (Driedonks et al. 2015). Besides, we cannot exclude that the thermo-tolerance trait might be linked with unfavourable traits that were selected against during domestication lowering the stress tolerance of domesticated tomato. (ii) High pollen viability is not necessary to the survival of tomato in its natural habitats, or it is not the only strategy to withstand extreme heat. In the screening presented in Chapter 5, we observed that some wild species of tomato accumulated a large number of pollen with a rather low pollen viability (e.g. LA1580). It is tempting to speculate that the production of higher numbers of cells might be energetically cheaper for a plant than producing a larger proportion of viable cells filled with protective metabolites (e.g. sugars, antioxidant). Hence, the low fraction of remaining viable pollen might be enough to produce seeded fruit and ensure the survival of a population – the task for which the plant generative system originally evolved for in nature, and later has been put at the service of feeding mankind. (iii) To enlarge genetic diversity under unfavourable conditions – The female gamete is known to be less vulnerable to high temperatures (Zinn et al. 2010). We observed that under heat stress conditions, flowers can present a stigma extension (data not-shown) that might impair the self-pollination of the flowers which is the common reproduction mechanism of tomato. Hence, the low production of viable pollen cells might cost less to the plant than promoting cross-fertilization which at the same time can prevent the fixation of genes and maintain a greater genetic diversity (Hedhly et al. 2005).

To conclude our results show that we can design ways to improve the thermo-tolerance of plants with the aim to safeguard fruit production under changing climate conditions. This research not only offers possibilities for such improvements in tomato but due to its general nature also for improvement in other crops.

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Summary

Tomato, the second most produced vegetable of the world, is threatened by climate change and more specifically the accompanying heat wave increases. The tomato production is highly dependent on temperatures since high temperatures lead to a decrease of pollen fertility and therefore fruit set. Evidence is accumulated that the loss of fertility is linked to an alteration of the pollen metabolome. However, most of the studies on pollen were performed using targeted biochemical approaches. We used untargeted metabolomics approaches such as LC-MS to broaden the detection of pollen metabolites and metabolites associated with the tolerance to high temperatures.

At first, we reviewed the state of the art of the pollen metabolome during development and under heat stress, we confirmed the association of sugars, flavonoids and polyamines in the protection to high temperatures (Chapter 2). We hypothesised that using untargeted metabolomics analysis will allow to discover more metabolites associated with pollen thermotolerance that can be used as metabolic marker in breeding program. Since metabolomics analysis are not common approaches in pollen we focused on the reliability of standard pollen isolation method applicable to metabolomics analysis. The main concerns of the reliability were based on the rehydration of dry pollen during isolation procedures, contamination from anther tissue and unwanted enzymatic activities during sample preparation (Chapter 3). We have shown that (i) isolating pollen in a solution leads to pollen rehydration and metabolic changes including the amino acid serine, glutamine glutamate, and the phenol kaempferol glucoside rhamnoside, its aglycone form and the 5-caffeoyl quinic acid, (ii) despite the filtration and washing steps the pollen sample isolated with anther squeezing is not exempt of anther contamination (e.g. alkaloids) and (iii) that lyophilising rehydrated pollen prevents sugar conversion that can occur during metabolite extraction. From this study, we have concluded that the current methods used to assess the pollen metabolome are suboptimal, but necessary to isolate young microspores. Therefore, effort should be put in attempts to develop an isolation method that prevents the drawbacks of current used methods, and that pollen isolated in solution should be qualified as imbibed since its metabolic state differs from the one of dry pollen. We have applied the method used in Chapter 3, by limiting the incubation time in solution and preventing sugar conversion, to assess the secondary metabolic profile of developing tomato pollen (Chapter 4) under control and short heat stress. We used a short heat stress to prevent pollen from undergoing physiological death and assessed changes that might be associated with acquired thermotolerance. We found that developing pollen accumulate a high abundance of flavonoids and showed a slight decrease of polyamines. The short heat stress did not lead to a strong metabolic response since only the total abundance of flavonoids were affected by the heat stress. Hence, we decided to use a longer but milder heat stress to determine the metabolites that might be associated with pollen thermotolerance. We first screened under high temperatures different tomato genotypes for pollen quality by recording pollen numbers and pollen viability (Chapter 4). We found that pollen numbers and pollen viability are not associated but that a combination of both traits might be an advantage to ensure fertilization under high temperatures. From the screening, we selected M82 as sensitive genotype and Nagcarlang as tolerant genotype. Instead of pursing our work on pollen we chose to assess the metabolome of the whole anther, since under short heat stress the pollen showed a weak response while we had evidence that the metabolome of anthers was strongly affected by short

heat stress. We first determined which developmental stage is the weakest to high temperatures and lead to the drop of pollen viability of M82 (Chapter 5). We found that in both genotypes the pollen development was delayed under high temperatures, but that in M82 the pollen viability started to drop during microsporogenesis and was at its minimum at polarization stage whereas in Nagcarlang such a drop did not occur and the polarization was faster than under control condition. We found that the drop of pollen viability might be due to a low amount of hexoses that might alter the availability of energy required under heat stress, and a lower antioxidant and osmolyte protection (e.g. flavonoids, polyamines and proline). We were able to confirm known metabolites to be associated with the tolerance such as the sugars and the amino acid proline, and to detect new metabolites that might be associated with the tolerance to high temperatures such as specific flavonoids, polyamines and the alkaloids. After confirmation of these metabolites as general metabolites associated with tolerance, we suggest their use as markers in a breeding program to develop and identify genotypes tolerant to high temperatures, that might ensure the production of tomato fruits under global warming conditions.

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Education certificate

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Marine Paupière
Date: 12 June 2017
Group: Laboratory of Plant Breeding
University: Wageningen University & Research

1) Start-up phase	date
► **First presentation of your project**	
Title: Genetical Metabolomics of thermotolerance in tomato pollen	26 Aug 2013
► **Writing or rewriting a project proposal**	
Title: Genetical metabolomics of thermotolerance in tomato pollen	Feb 2013
► **Writing a review or book chapter**	
The metabolic basis of pollen thermo-tolerance: Perspectives for breeding', Metabolites 2014, 4, 889-920; doi: 10.3390/metabo4040889	Sep 2014
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.0 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day, Leiden, NL	29 Nov 2013
EPS PhD student days 'GET2GETHER', Soest, NL	28-29 Jan 2016
► **EPS theme symposia**	
EPS Theme 3 Symposium 'Metabolism and Adaptation', Amsterdam, NL	22 Mar 2013
EPS Theme 3 Symposium 'Metabolism and Adaptation', Wageningen, NL	11 Mar 2014
EPS Theme 3 Symposium 'Metabolism and Adaptation', Utrecht, NL	10 Feb 2015
► **Lunteren days and other National Platforms**	
Annual meeting 'Experimental Plant Sciences' , Lunteren, NL	22-23 Apr 2013
Annual meeting 'Experimental Plant Sciences' , Lunteren, NL	14-15 Apr 2014
Plant Breeding - research day	24 Sep 2014
► **Seminars (series), workshops and symposia**	
Workshop I Anther Development under Heat Stress - Radboud University (SPOT-ITN), Nijmegen, NL	07-13 May 2013
Workshop II Analyses of Heat Stress Response Factors - Frankfurt University (SPOT-ITN), Germany	24-29 Mar 2014
Workshop III proteomics - Vienna University (SPOT-ITN), Austria	03-07 Nov 2014
Symposium 'Omics advances for academia and industry'	11 Dec 2014
► **Seminar plus**	
► **International symposia and congresses**	
Next Generation Plant Breeding Conference, Ede, NL	11-14 Nov 2012
1st SPOT-ITN Symposium, Frankfurt, Germany	28-29 Jan 2013
5th European Plant Science Retreat, Gent, Belgium	23-26 Jul 2013
SPOT-ITN mid-term meeting, Arnhem, NL	04-08 Nov 2013
The XXIIIth International Sexual Plant Reproduction, Porto, Portugal	13-18 Jul 2014
Stress Biology and Criop fertility conference, Sorento, Italy	18-21 Mar 2015
► **Presentations**	
Talk - 1st SPOT-ITN Symposium, Frankfurt, Germany	28-29 Jan 2013
Poster - 1st SPOT-ITN Symposium, Frankfurt, Germany	28-29 Jan 2013
Poster - 5th European Plant Science Retreat, Gent, Belgium	23-26 Jul 2013
Talk - SPOT-ITN mid-term meeting, Arnhem, NL	04-08 Nov 2013
Poster - SPOT-ITN mid-term meeting, Arnhem, NL	04-08 Nov 2013
Poster - The XXIIIth International Sexual Plant Reproduction in Porto	13-18 July 2014
Guest Talk - Naples University	02 Dec 2014
Talk - Stress Biology and Criop fertility conference in Sorento	18-21 Mar 2015
Poster - Stress Biology and Criop fertility conference in Sorento	18-21 Mar 2015
Talk - PhD Student Days 'GET2GETHER'	29 Jan 2016
► **Excursions**	
Excursion to Nunhems B.V	May 2013
Excursion to company in Wageningen	Nov 2013
Subtotal Scientific Exposure	*25.1credits**

CONTINUED ON NEXT PAGE

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Basic statistics	18-26 Jun 2013
Transcription factors and transcriptional Regulation	17-19 Dec 2013
Systems biology "Statistical analysis of -omics data"	15-19 Dec 2014
► **Journal club**	
► **Individual research training**	
Bioinformatics, lab of Hamed Bostan (SPOT-ITN), Naples, Italy	29 Nov-06 Dec 2014
Subtotal In-Depth Studies	*5.5 credits**

4) Personal development	*date*
► **Skill training courses**	
Scientific Integrity	05 Jun 2013
Philosophy and Ethics of Food Science and technology	Jan-Feb 2014
Techniques for writing and presenting a scientific paper	26-29 Aug 2014
Scientific publishing	09 Oct 2014
Efficient Writing Strategies	Oct-Nov 2014
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4.4 credits**

TOTAL NUMBER OF CREDIT POINTS*	**42.0**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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