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Dissecting Molecular Mechanism of Heat Stress on Anther Development in Fragaria Vesca

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DISSECTING MOLECULAR MECHANISM OF HEAT STRESS ON ANTHER DEVELOPMENT IN *FRAGARIA VESCA*

by

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ABSTRACT

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Under the Supervision of Prof. Dazhong Zhao

Strawberry is an important fruit crop in the Rosaceae family. Originally from the temperate region, strawberry is vulnerable to heat waves, which reduce fruit yield and quality. Previous studies have shown that heat stress impairs pollen development; however, the molecular mechanisms by which heat stress affects the development of anthers, where pollen develops, are unclear. Due to the genome complexity of cultivated strawberry (*Fragaria* × *ananassa*, 2n = 8x= 56), *Fragaria vesca* (2n = 2x = 14) was used, which emerges as a diploid model plant for Rosaceae, to study the effects of heat stress on anther development at morphological and molecular levels. In this study, a complete anther development series was established, which helped identify key stages affected by heat stress via defining morphological hallmarks at each developmental stage. It was demonstrated that heat stress led to male sterility in *Fragaria vesca* by affecting anther development at two key stages- meiotic stage-6 and post-meiotic stage-8. Morphological changes of anther cell differentiation caused by heat stress at these two key stages were further revealed using backscattered scanning electron microscopy imaging. Finally, a stage-specific transcriptomics approach was utilized to study the underlying molecular mechanisms of responses to heat stress. Heat stress altered expression of many genes, including those encoding heat shock proteins, transcription factors, genes involved in cell wall, and histone modification enzymes. My thesis work lays the groundwork for future discoveries in heat stress

associated with male sterility within the Rosaceae family. The genes identified as key players in response to heat stress can be targeted in efforts to improve heat tolerance in crops.

To
My Parents and Brother

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Chapter 1

Introduction

1. Strawberry Background

Strawberry (*Fragaria* x *ananassa* Duch.) is one of the most important fruit crops that is widely planted in the United States. US ranks second in strawberry production worldwide. The US strawberry industry generated $2.67 billion of economic value in 2018 (https://www.nass.usda.gov/Publications/Todays_Reports/reports/ncit0619.pdf), making it No. 3 after grape and apple among non-citrus fruits. In the US, strawberries are produced in 8 regions, i.e. Region 1: California, Region 2: Pacific Northwest (Oregon and Washington), Region 3: Upper Midwest (Iowa, Michigan, Minnesota, North Dakota, South Dakota, and Wisconsin), Region 4: Lower Midwest (Illinois, Indiana, Kansas, Missouri, Nebraska, and Ohio), Region 5: Mid-South (Arkansas, Louisiana, Mississippi, Oklahoma, and Texas), Region 6: Northeast (Connecticut, Delaware, Maine, Maryland, Massachusetts, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont, and West Virginia), Region 7: South Atlantic (Alabama, Georgia, North Carolina, South Carolina, and Virginia), and Region 8: Florida (Samtani *et al.*, 2019). In 2017, California and Florida produced an estimated 91% and 8% of total strawberries, respectively, while only 1% of strawberries were produced in the other 6 regions combined although by 85% of farms (>9000) using about 17% of acreage (USDA, 2018). Due to various factors such as abiotic stresses caused by climate changes, the acreage in California has declined in recent years (USDA, 2017, 2018). The public has become more interested in local foods and the year-round consumption of strawberries keeps growing yearly.

Therefore, to meet the increasing demands, it is imperative to develop new cultivars to expand strawberry production in both traditional and new regions.

As a temperate crop, the optimum growth temperatures for strawberries range from 10°C to 26°C (Kadir *et al.*, 2006; Kumakura and Shishido, 1995; Ledesma *et al.*, 2008; Ledesma and Sugiyama, 2005; Pipattanawong *et al.*, 2009). Long-term high-temperature stress decreases flower numbers but promotes runner formation in strawberries (Darrow, 1966; Durner, 1988; Slovin and Rabinowicz, 2007). Short-term high-temperature stress (acute heat stress, called heat stress from this point forward) during flowering time not only reduces fruit size, weight, and flavor, but also causes deformed fruits with poor coloration (Durner, 1988; Kadir *et al.*, 2006; Kumakura and Shishido, 1994; Ledesma *et al.*, 2008; Ledesma and Sugiyama, 2005; Miura *et al.*, 1994; Mori, 1998; Wang and Camp, 2000; Went, 1957; Winardiantika *et al.*, 2014). Although different cultivars respond to heat stress differently during the reproductive stage, analysis of seven strawberry cultivars under high temperatures in the field showed that high stress decreased fresh weight of fruits in all tested cultivars, but to different degrees (Noguchi and Yamakawa, 1988). When daytime growing temperatures for the Nyoho cultivar increased from 25°C to 30°C and to 35°C during harvest time, the fresh fruit yield per plant decreased in succession (Kumakura and Shishido, 1995). Under heat stress [30°C/25°C (day/night, the same thereafter) for 2 days], the heat sensitive cultivar Toyonoka had much lower fruit set in comparison with the heat insensitive cultivar Nyoho, although numbers of inflorescences and flowers, as well as fresh weight in all fruit positions in both cultivars were reduced (Ledesma *et al.*, 2008). Moreover, heat stress at 30°C/22°C reduced fruit size and weight in both Earliglow and Kent cultivars; with the increasing of temperatures, the contents of soluble solids (SSC), fructose, sucrose, glucose, and total carbohydrates were decreased, thus affecting fruit quality

(Wang and Camp, 2000). Even under 42°C for 4 hours both Nyoho and Toyonoka had significantly reduced fruit set and fruit size; however, the effects of this short but severe heat stress vary with flower stages, flower positions, and cultivars (Ledesma and Kawabata, 2016). Collectively, short-term (like a single day) heat stress, which often occurs in the field and greenhouse, can severely impair fruit yield and quality in strawberry.

Traditionally, strawberries are cultivated in regions with suitable climate conditions. Depending on the region, short-day cultivars produce fruits during spring or summer season, thus they are also called spring-bearing or June-bearing cultivars (Durner *et al.*, 1984). Day-neutral cultivars produce flowers and buds throughout the season; however, high temperatures (>30°C) restrain flowering and fruiting (Durner *et al.*, 1984). In California, both day-neutral and short-day cultivars are grown. Both types of cultivars are propagated in Northern California. Summer in Southern California or even in central Southern California is too warm to allow acceptable strawberry production. The demand for local strawberries is steadily increasing in Texas (Hinson and Bruchhaus, 2008); however, only about 0.5% of total strawberries are produced in the Mid-South where high temperatures during harvest season limit the production duration for both short-day and day-neutral cultivars. A similar issue also exists in other strawberry production regions in the US.

All regions are facing various challenges, although many new technologies and management tools have been used to maximize strawberry production all year around. Strawberry is a temperate crop whose flowering and fruiting season coincide with temperature increase from winter to spring to early summer; thus, high temperatures due to seasonal changes and global warming in most strawberry producing areas often inhibit fruit production in terms of yield, quality, and harvest duration. Using protected culture, such as low tunnel, high tunnel, and

shade cloth, becomes a common trend for strawberry production. Cultivation using low and high tunnel can advance harvest time, while shading extends harvest season; however, temperatures in the tunnels (>34°C) are too high for all cultivars to develop edible, tasteful, and large fruits in late spring or early summer (Kadir *et al.*, 2006). Besides other factors, developing and using heat-tolerant cultivars are the most effective ways to avoid damage from heat stress in the open field and greenhouse. Moreover, heat-tolerant cultivars will bring revolutionary changes to the strawberry industry, since they not only can be used to extend harvest season, but also can be cultivated in warm regions and non-traditional areas, which therefore could greatly increase strawberry production. To breed heat-tolerant cultivars, it is critical to determine how short-term heat stress impairs fruit development during blossom at the morphological and molecular levels.

1.1a. *Fragaria vesca* - a model plant to study Rosaceae family

Rosaceae is one of the largest flowering plant families, comprising over 100 genera and 3000 species of herbs, shrubs, and trees in diverse geographical locations, such as North America, Europe and Asia (Folta and Davis, 2006). A variety of economically significant fruit and nut crops that belong to the *Rosaceae* family includes strawberry (*Fragaria x ananassa*), apple (*Malus domestica*), raspberry (*Rubus*), blackberry (*Rubus fruticosus*), cherry (*Prunus avium*), plum (*Prunus domestica*), peach (*Prunus persica*) pear (*Pyrus communis*), and almond (*Prunus dulcis*) (Dirlewanger *et al.*, 2002; Shulaev *et al.*, 2008). Most of the major fruit trees in the *Rosaceae* are perennial woody plants, unlike strawberry.

The strawberry is an herbaceous perennial plant. *Fragaria* × *ananassa* (2n = 8x= 56) is the cultivated strawberry that contributes greatly to the fresh fruit market. The herbaceous nature

of strawberry makes it easier to engineer at the genetic level than the woody plant members of the family are.

One of the progenitors of *F. x ananassa* is *Fragaria vesca*. Four subspecies of *F. vesca* originated from different regions: ssp. *vesca* from Europe, ssp. *americana* from North America east of the Rocky Mountains, ssp. *bracteata* from North America west of the Rocky Mountains, and ssp. *californica* from the central California coast (Folta and Davis, 2006). *F.* vesca is self-fertile and can yield large amounts of seeds per fruit (Ahmadi and Bringhurst, 1991). *F. vesca* has a short life cycle of about 3-4 months. Compared with the octaploid *Fragaria* × *ananassa* (2n = 8x= 56) whose genome is estimated as 708-720Mb (Akiyama *et al.*, 2001), the diploid *F.vesca* (2n = 2x = 14) has a smaller genome (240 Mb) (Shulaev *et al.*, 2011). Therefore, *F. vesca* emerges as an important system for molecular genetics and genomic studies for fruit plants in *Rosaceae* (Hollender *et al.*, 2014).

Fragaria vesca is a runner-less perennial plant. The plant has elongated petioles bearing trifoliate leaves and determinate dichasial cyme inflorescence (Anderson *et al.*, 1982; Hancock, 1999; Hollender *et al.*, 2012). The flowers in the inflorescence represent basipetal succession, where the oldest flower is at the top, and the youngest flowers are at the base. The primary shoot forms the primary flower that gives rise to the first fruit. Further, subtending the primary flower are two secondary shoots opposite to each other terminating as secondary flowers. Lastly, each secondary flower also bear two tertiary flowers opposite to each other (Hollender *et al.*, 2012) (Fig. 1.1 A, B).

An individual *F. vesca* flower consists of 5 whorls (Fig.1.2 A, B). The outermost whorl has five bracts that alternate in position to five green leafy sepals in the second whorl. Interior to the sepals are five white petals in the third whorl. Following these are the two reproductive

whorls, comprised of male reproductive organs – the stamens and female reproductive organs – the pistils, respectively. In the fourth whorl, there are twenty stamens, which are categorized as short (S), medium (M), and tall (T) stamens. Ten medium stamens are arranged near the petals, whereas five tall and five short stamens are arranged alternately near the central receptacle. The innermost whorl comprises a dome shaped structure, which has approximately 160 pistils arranged in a spiral manner on each receptacle (Hollender *et al.*, 2012). Each pistil comprises one ovary and one ovule that, upon fertilization by pollen, gives rise to an achene- the true fruit of strawberry.

Strawberry propagates asexually through runners and branch crowns. Fruit formation in strawberry depends on successful sexual reproduction, which involves self-fertilization, and consequently seed development. Ideally the fruit produced in strawberry is a modified stem tip (Darrow, 1966; Hancock, 1999). In botanical terms strawberry fruit represents a receptacle, which bears achenes. These achenes appear as dots on the surface of the fleshy receptacle. Auxin plays an indispensable role in strawberry fruit formation, fertilized achenes secrete auxin, which further leads to enlargement of the receptacle (Nitsch, 1950). Removal of achenes from the receptacle causes deformed fruit formation, while exogenous application of auxin rescues the fleshy fruit (Nitsch, 1950). Thus, successful fertilization and pollination is required for proper fruit development in strawberry. The importance of auxin in fruit formation has been recently elucidated (Kang *et al.*, 2013).

Interestingly, application of N-1-naphthylphthalamic acid (NPA), a chemical inhibitor of auxin transport, led to receptacle enlargement. The presence of NPA inhibits the function of PIN proteins, leading to sequestration of IAA (Indole-3-Acetic Acid) in the ovule itself so that it is not transported out of the ovary walls. This suggests a role for increased use of artificial auxin in

fruit formation (Kang *et al.*, 2013). Furthermore, exogenous application of gibberellin (GA) onto emasculated *F. vesca* flowers led to fruit enlargement without involvement of fertilization events. Also, wild-type fruit size was obtained when combined application of auxin (NAA) and gibberellin (GA) was used, hence indicating the importance of both hormones during fruit development (Kang *et al.*, 2013). Transcriptome study of different developmental stages of strawberry fruit helped in unraveling the molecular mechanisms associated with its fruit set. It was reported that for fruit set, endosperm and seed coat play an important role in auxin and gibberellin biosynthesis pathways. Some of the auxin biosynthesis genes expressed in the achenes include *YUC5, YUC11* an*d TAR1*. Gibberellin biosynthesis genes expressed include *GA20ox3, GA3ox3, 4, 5, and 6* (Kang *et al.*, 2013). This study reported temporal and spatial regulation of hormonal regulation in fruit development. However, how auxin genes control this phenomenon still remains unclear.

2. Heat stress in plants

Heat stress is one of the most damaging abiotic stresses to plants. It causes irreversible damage to different parts of the plant, which further affects overall yield. The response of plants to heat stress varies with duration, magnitude, and the plant type. Heat stress has detrimental effects on plant growth and development in general, such as scorching of leaves and stems, shoot and root growth inhibition, yield loss, and fruit damage. Altered membrane stability, protein folding, and reactive oxygen species production are some of the direct consequences of heat stress (Hasanuzzaman *et al.*, 2013; Kotak *et al.*, 2007). Leaky membranes due to heat stress inhibit the transport of photosynthates (Mohammed and Tarpley, 2010). Additionally, it also affects the proper functioning of the photosynthetic apparatus (Wang *et al.*, 2009). Specifically, under high

temperature, loss of grana stacks and swelling of grana alter the thylakoid structure in the photosynthetic machinery. This further leads to abnormal photochemical reactions (Karim *et al.*, 1997; Wahid *et al.*, 2007; Wise *et al.*, 2004).

Plant sexual reproduction is highly sensitive to heat stress. Aberrant pollination, unviable pollen grains, low pollen germination rate, dysfunctional fertilization, as well as delayed growth of floral buds, flowers and fruits are direct effects of high temperature on reproduction, which ultimately result in significant yield loss (Abiko *et al.*, 2005; Arshad *et al.*, 2017; Begcy *et al.*, 2019; Endo *et al.*, 2009; Giorno *et al.*, 2013; Harsant *et al.*, 2013; Jagadish, 2020; Janni *et al.*, 2020; Ma *et al.*, 2020; Pressman *et al.*, 2002; Sakata *et al.*, 2000; Zinn *et al.*, 2010). However, how heat stress affects development of female tissue remains unknown, as very few studies have been performed on the female reproductive organs during heat stress. For instance, effects of heat stress on ovary development in wheat resulted in abnormal nuclei and embryo sac (Saini *et al.*, 1984). Some of the well-studied examples of the crops affected by high temperature during male gametophyte development include common bean (*Phaseolus vulgaris* L.) (Konsens *et al.*, 1991), cowpea (Ahmed *et al.*, 1992), tomato (*Solanum lycopersicum*); (Levy *et al.*, 1978), and cotton (*Gossypium hirsutum*) (Reddy *et al.*, 1992). Previous studies were mostly focused on the effects on heat stress on late processes of sexual reproduction; however, how heat stress affects development of male and female reproductive organs is largely unclear.

2.1 Plant male reproduction is highly sensitive to heat stress

Heat stress impairs anther development, microsporogenesis, and pollen formation, which causes partial or complete male sterility in various plants (Table 1.1). The stamen is the male reproductive organ of flowering plants, comprised of an anther where pollen (the male gametophyte) develops and a filament that anchors the anther to the flower. A typical anther has four lobes (microsporangia) (Feng *et al.*, 2013; Goldberg *et al.*, 1993; Walbot and Egger, 2016; Zhao, 2009); within each lobe, the central pollen mother cells (PMCs) or microsporocytes, are surrounded by four concentrically organized layers of somatic cells: the epidermis, endothecium, middle layer, and tapetum (outside to inside). PMCs give rise to pollen via a series of complex events. Each PMC undergoes meiosis to produce a tetrad of microspores enclosed in a callose wall. Dissolution of the callose releases the microspores into the locule. After two rounds of mitosis, microspores eventually become binucleate pollen grains which contain a vegetative and a generative cell (Sanders *et al.*, 1999) (Fig. 1.3). The somatic anther wall cells, particularly tapetal cells (tapetum) are essential. Tapetum, consisting of a monolayer or multilayers of endopolyploid cells, is associated with successive stages of PMC, tetrads, microspores, and developing pollen as anther development progresses (Goldberg *et al.*, 1993; Scott *et al.*, 2004; Walbot and Egger, 2016) (Fig. 1.3). Early on, tapetal cells secrete enzymes required for releasing haploid microspores from tetrads (Clément and Pacini, 2001; Hsieh and Huang, 2007; Ishiguro *et al.*, 2010; Pacini *et al.*, 1985; Parish and Li, 2010). Later, tapetal cells provide energy and materials for pollen development and pollen coat formation (Huang *et al.*, 2017; Parish and Li, 2010; Wang *et al.*, 2003; Wu *et al.*, 1997). Lack of tapetum or an abnormal tapetum impairs microspore and pollen development, causing male sterility (Mariani *et al.*, 1990; Zhang *et al.*, 2014; Zhao *et al.*, 2002). The middle layer is important for pollen development, and anther

dehiscence (Cecchetti *et al.*, 2017; Mizuno *et al.*, 2007). In addition, the endothecium is also necessary for anther dehiscence (Cecchetti *et al.*, 2013; Murphy *et al.*, 2015).

Heat stress-induced male sterility and seed yield loss are mainly ascribed to aberrant tapetum and pollen development (De Storme and Geelen, 2014; Parish *et al.*, 2012, Rieu *et al.*, 2017). Decreased pollen viability due to heat stress has been reported in many crops, such as common bean (Gross and Kigel, 1994; Prasad *et al.*, 2002), rice (Endo *et al.*, 2009), cotton (Song *et al.*, 2015), tomato (Pressman *et al.*, 2002), pepper (Erickson and Markhart, 2002), wheat (Anjum *et al.*, 2008; Oshino *et al.*, 2011), and flax (Cross *et al.*, 2003). In crops such as wheat, episodes of male sterility were observed upon 3 days of treatment at 30°C/30°C (day/night, same from this point forward) during meiosis, and further studies revealed that abnormal tapetum degeneration is a plausible cause for pollen sterility (Saini *et al.*, 1984). Moreover, wheat PMCs display abnormal meiosis during heat stress (Omidi *et al.*, 2014). Premature pollen development in common bean at 33°C/29°C is also a result of early degenerated tapetal cells (Suzuki *et al.*, 2001). Apart from tapetal cell degradation in barley, moderately high temperatures of 30°C/25°C leads to abnormalities in mitochondria, rough endoplasmic reticulum, and nuclear membranes in PMCs (Oshino *et al.*, 2007). Abnormally wavy, looped rough endoplasmic reticulum (ER) structures were observed in heat-stressed tapetal cells (Suzuki *et al.*, 2001), suggesting that ER irregularities and malfunction in tapetal cells might cause male sterility (De Storme and Geelen, 2014). Additionally, DNA fragmentation, cytoplasmic shrinkage, and vacuolation were observed in early tapetal cells of thermosensitive genic male-sterile (TGMS) rice due to heat stress. This indicates that the precocious programmed cell death (PCD) of tapetal cells during heat stress further results in male sterility (Ku *et al.*, 2003). Disruption of tapetal cells during heat stress can also result in aberrant callose degradation in PMCs and aberrant pollen wall formation, such as

disruption of exine patterning, leading to male sterility (Djanaguiraman *et al.*, 2014; Parish *et al.*, 2012; Suzuki *et al.*, 2001).

Anther wall cells and pollen grains in tomato plants upon heat stress (32°C/26°C) exhibit decreased starch and soluble sugar contents (Pressman *et al.*, 2002). Similarly, heat-stressed (36°C/26°C) sorghum microspores also showed reduced starch content and sucrose deficiency (Jain *et al.*, 2007). Additionally, an imbalance in reactive oxygen species (ROS) homeostasis in tapetal cells resulting from heat stress possibly leads to early programmed cell death PCD of tapetal cells (De Storme and Geelen, 2014). The defects induced in anther cells such as tapetum involve misregulation of tissue specific genes. For example, in rice, tapetum-specific genes such as *YY1* and *YY2* (*AK107918* and *AK105510*, respectively) were repressed by heat stress (Endo *et al.*, 2009). Likewise, in barley high temperature causes up-regulation of the meiosis-specific gene *Asy1* (Oshino *et al.*, 2007). One of the possible molecular mechanisms behind the male sterility in barley is attributed to hyper phosphorylation of Ser-5 residue of C-terminal domain of RNA polymerase II, which alters gene expression during heat stress (Abiko *et al.*, 2005). Moreover, hormones are also well known to play a significant role during high temperature stress. Auxin synthesis in *Arabidopsis* and barley anthers is reduced during high temperatures, whereas exogenous application of auxin to anthers improved pollen thermotolerance in barley (Higashitani, 2013; Sakata *et al.*, 2010). Furthermore, auxin biosynthesis genes such as *YUC2* and *YUC6* were suppressed in anthers exposed to high temperatures (33°C) (Sakata *et al.*, 2010). Various studies showed that male reproduction, mainly male meiosis, microsporogenesis, and anther dehiscence, is highly susceptible to heat stress; however, the underlying molecular mechanisms remain largely unknown.

2.2 Heat stress response in plants at transcriptional and translational level

A change in temperature is sensed by various mechanisms, which leads to changes in heat responsive gene expression (Mittler *et al.*, 2012). At the transcriptional level, heat shock transcription factors (HSFs) help in regulating downstream signaling. HSFs promote expression of heat shock proteins via binding to conserved Heat Shock Elements (HSE) in the promoter region of HS responsive genes. HSPs help restore protein homeostasis by functioning as molecular chaperons. Denaturation and aggregation of cell proteins in response to high temperatures is prevented by HSPs via assisting refolding of the denatured proteins (Frydman, 2001; Janni *et al.*, 2020; Ohama *et al.*, 2017; Scharf *et al.*, 2012). Secondly, unfolded proteins that are induced by high temperatures are sensed *via* thermosensors localized in the cytoplasm and ER which are part of the unfolded protein response (UPR) (Moreno and Orellana, 2011). Different factors within the UPR have been found to contribute to the heat stress response including *HEAT SHOCK FACTOR A2 (HSFA2)*. *HSFA2*, one of the regulators of the heat stress response, is a component of cytoplasmic protein response (Sugio *et al.*, 2009). Additionally, endoplasmic reticulum UPR is regulated by release of transcriptional factor Basic Leucine Zipper (*bZIP*) into the nucleus for expression of heat induced genes (Deng *et al.*, 2011). Moreover, ROS accumulation forms a part of the heat stress response. In normal conditions ROS are released in mitochondria, chloroplast, and peroxisomes whereas, during high temperatures ROS detoxification and production is unbalanced and leads to accumulation inside the cell (Sharma *et al.*, 2012). Some of the genes studied during heat stress are *HSP101* (heat shock protein 101) in maize (Young *et al.*, 2001), carrot *shSP17.7* (small heat shock protein 17.7) (Hu *et al.*, 2010), *APX1* (ascorbate peroxidase 1) in *Arabidopsis* (Suzuki *et al.*, 2013), and *AChE* (acetylcholine enhancer) in maize (Yamamoto *et al.*, 2011) (listed in Table 1). There are certain

transcription factors involved during heat stress such as elongation factor (EF-Tu), basic helix-like-helix (bHLH), and phytochrome interacting factors (*PIFs*) (Leivar and Quail, 2011) (Listed in Table 1.2).

2.2a Pathways responding to heat stress during male reproduction

Heat stress is one of the detrimental abiotic stresses that cause irreversible damage to crops. Increasing demand for crop yield requires healthy and undamaged crops. Thus, there is a need for the identification of pathways or genes that can help in engineering thermotolerant crops to sustain productivity despite episodes of heat stress.

In order to study the effect of high temperature on male reproductive development at the transcriptomics level, microarray and RNA-seq approaches have been widely used in different plants. Some well-studied plants have been reviewed in Table 1.3. These approaches have led to identification of key pathways and genes associated with male reproductive development during heat stress. Some of the important crops listed here include tomato (Bita *et al.*, 2011), maize (Begcy *et al.*, 2019; Casaretto *et al.*, 2016), and cotton. Tomato is one of the most studied model plants for heat stress. Transcription analysis was performed on heat stressed (43–45 °C for 2 h) tomato anthers using microarray hybridization (Frank *et al.*, 2009). The result included enrichment of heat shock proteins, and heat shock factors, along with ROS scavenging genes such as *ASCORBATE PEROXIDASE* (*APX1*, *APX2*) that were found to be highly expressed in the microspores during heat stress. Moreover, the data indicated the presence of transcripts of ethylene-responsive genes such as *ETHYLENE-RESPONSIVE LATE EMBRYOGENESIS-LIKE PROTEIN* (*ER5* and *ER21*) in pollen grains (Frank *et al.*, 2009).

In the cereal crop rice, transcriptomics data from anther, upon exposure to heat stress (38°C/ 28°C day/night), observed transcripts associated with metabolic processes. Transcription factors such as MYB, F-box, AP2/ERF superfamily, bZIP, and WRKY were also found. Furthermore, expression of the above-mentioned genes in anther tissues was highly down regulated due to heat stress (Liu *et al.*, 2020). Apart from the above-mentioned category of transcription factors, processes such as nucleic acid synthesis, hormone signaling pathways, photosynthesis, and protein synthesis and modification were also misregulated in anthers during heat stress (Liu *et al.*, 2020). Additionally, the transcriptomics profile of heat-stressed sorghum microspores indicated misregulation of genes involved in sucrose cleavage, transport and utilization (Jain *et al.*, 2007), thus indicating that heat stress leads to extensive transcriptional regulation in anthers.

In barley, effects of short-term heat stress (42°C) on caryopsis development were also studied at the transcriptional level. The study reported reduced storage compound biosynthesis and cell growth as factors contributing to impaired caryopsis development (Mangelsen *et al.*, 2011).

In another important cash crop, i.e., cotton, RNA-seq data from heat-stressed anthers revealed cell growth and metabolic activities affected to an even greater extent. A lot of transcripts were found under categories such as protein processing, plant hormone signal transduction, carbohydrate metabolism, and endoplasmic stress (Min *et al.*, 2014).

Apart from transcriptomics studies, proteomic analysis is also utilized in studying the heat stress response. Proteomic studies on heat stress have focused on proteins extracted from the plant parts such as the leaves, grains, and fruits. However, not much has been studied using reproductive organs, such as anthers. The presence of heat-responsive proteins can further help

identify underlying mechanisms to combat episodes of high temperature occurring in the environment. Protein profiling provides much better insights than transcriptomics studies about the molecular mechanism associated with heat stress. Various plants have been used to explore the protein profiles during heat stress. For example, in rice, comparative proteomics was utilized to study the mechanism of heat stress in anthers of heat-resistant and heat-sensitive cultivars using isobaric tags for relative and absolute quantitation (iTRAQ). Protein profiling of the sensitive cultivar demonstrated degradation of ribosomal proteins due to heat stress, whereas heat stress led to increased expression of sHSP, β-expansins and lipid transfer proteins in the case of the resistant cultivar. The expression of these genes in the resistant cultivar thus indicates their importance in providing tolerance to heat stress (Mu *et al.,* 2017). Moreover, the anthesis stage in rice is most susceptible to heat stress. Proteomics data on N22, a heat-tolerant genotype, shows induction of cold and heat shock proteins upon heat stress at 38°C, whereas, in the heat sensitive genotype Moroberekan, heat shock proteins were down regulated (Jagadish *et al.*, 2009). Similar to this, another proteomic study was performed on heat stressed rice anthers using shotgun proteomic analysis. This study identified about 139 differentially expressed proteins. Some of the highly expressed or induced proteins included heat shock, DnaK family, and chaperone proteins. Moreover, increased levels of trehalose synthase in anthers suggests a possible role for it in protection from protein denaturation during heat stress (Kim *et al.*, 2015b).

Shotgun proteomics (GEL-LTQ-Orbitrap MS) was also used to identify unique proteins in two different stages of tomato pollen during heat stress i.e., post-meiotic and mature. Data observed at the post-meiotic stage of anther development indicated the presence of late embryogenesis abundant protein (LEA) and small heat shock proteins (HSP 20 and HSP 22) in heat stress conditions, indicating their role in protection. The authors concluded that the presence

of low molecular weight HSPs plays an important role in development and maintaining cell membrane integrity in the microspore and polarized microspore stage. In the case of mature pollen, the proteins induced during heat stress were primarily involved in glycolysis and the TCA cycle. Some of them are ATP synthase (Solyc11g039980), mitochondrial ATP synthase (Solyc00g009020), citrate synthase (Solyc01g073740), ATP-citrate lyase A-2 (Solyc05g005160), Pyruvate dehydrogenase E1 component subunit beta (Solyc06g072580), and UTP-glucose1 phosphate uridylyltransferase (Solyc05g054060). The data suggests the involvement of different heat responsive genes associated with mild heat stress (Chaturvedi *et al.*, 2015). Most of the proteome changes that have been observed were focused on heat response phases but did not consider the effectiveness of the thermotolerance in plants. Very few studies incorporated comparisons of heat-sensitive plants and tolerant varieties to identify specific factors relevant in obtaining higher thermotolerance.

All of the above examples show that both transcriptomics and proteomics study help identify various pathways associated with different crop studied. Some of the common occurring pathways include heat responsive transcription factors and proteins, hormone signaling pathways and sucrose metabolism. These studies will further help elucidate genes from different pathways associated with crops that could help create heat tolerant varieties to combat high temperature stress.

2.2b Heat shock factors (Hsfs): conventional players

Heat shock factors (Hsf) are conventional transcription factors, evoked during heat stress. Hsfs bind to the HEAT SHOCK ELEMENTS (HSE) of target genes, which activates heat stress inducible genes including those encoding Heat Shock Proteins (Hsps), consequently leading to heat stress response (Kotak *et al.*, 2007; Scharf *et al.*, 2012). HsfA1 and HsfA2 are two key regulators of heat shock response, where HsfA1 acts as a master regulator (Charng *et al.*, 2007). Moreover, heat stress induces a truncated isoform of HsfA2 that initiates its transcription by binding to the promoter region of *HsfA2* gene (Liu *et al.*, 2013). HsfB1 and HsfB2b are also crucial for heat stress as they aid in the repression of heat stress-inducible genes such as *HsfA2* and *HsfA7a* (Ikeda *et al.*, 2011).

The role of heat shock proteins and factors in male reproductive tissue during heat stress has been well studied in plants (Bita *et al.*, 2011; Frank *et al.*, 2009; Giorno *et al.*, 2010; Zhang *et al.*, 2017). Expression of *HsfA2* is highly induced in anther tissues by heat stress, which is linked to thermo-protection of anther tissues during harsh heat conditions (Giorno *et al.*, 2010). In tomato pollen, during episodes of heat stress HsfA2 acts as a coactivator of Hsfa1a. Restrained expression of HsfA2 leads to reduced pollen viability and germination rates. Anther stage-specific exposure to heat stress found that meiosis and microspore stages are heat sensitive where HsfA2 plays a role in heat stress response thermo-tolerant in *Solanum lycopersicon* (Fragkostefanakis *et al.*, 2016). Furthermore, pollen grains produce a higher amount of Hsps as compared to the vegetative tissues (Mascarenhas et al., 1996). HSPs protect misfolded proteins induced by heat stress (Baniwal *et al.*, 2004; Neumann *et al.*, 1989; Volkov *et al.*, 2005). To study the role of HSPs in providing thermotolerance during heat stress, transgenic lines were created. For example, overexpression of an HSP, *AtHSP101*, resulted in enhanced heat tolerance

of cotton and tobacco transgenic lines. Pollen grains from both transgenic lines exhibited higher germination rate along with pollen tube elongation during heat stress (Burke and Chen, 2015). This fine regulation of Hsfs and Hsps during male reproduction indicates that intense signaling take place to prevent male reproductive organs from damage caused by heat stress.

2.3 Epigenetic regulation of heat stress during male reproduction

Besides genetic regulation, epigenetic control, particularly DNA methylation, is an important mechanism for plants to cope with heat stress during male reproduction. RNA-directed DNA methylation (RdDM) in plants involves various components such as small interfering RNAs (siRNA) and the DNA methyltransferase *DOMAINS REARRANGED METHYLTRANSFERASE2* (*DRM2*), along with RNA polymerase, which regulates cytosine *de novo* methylation (Law and Jacobsen, 2010). Methylation of DNA occurs at specific sites: symmetric patterns of CpG/CpNpG and asymmetric CpNpN. There are specific enzymes which lead to targeted methylation; for example, *DNA METHYLTRANSFERASE 1* (MET1) and *DOMAINS REARRANGED METHYLTRANSFERASE 2* (DRM2) are involved in symmetric cytosine (CG) methylation (Cao 2003, Cao 2002). In plants, methylation of asymmetric cytosine (CpNpG) is regulated by *CHROMOMETHYLASE 1* (*CMT1*) (Bartee and Bender, 2001).

A pollen grain comprises one vegetative cell and two sperm cells which maintain more stable methylation patterns than leaves and roots (Hsieh *et al.*, 2016). The vegetative nucleus lacks the *DECREASE IN DNA METHYLATION 1* (*DDM*1) gene, leading to reactivation of transposable elements. Reduction of DNA methylation in shed pollen causes transcriptional reprogramming (Slotkin *et al.*, 2009). Cell specific DNA methylation studies revealed that CG and CHG methylation are retained in microspores and sperm cells, whereas the CHH

methylation is lost (Calarco *et al.*, 2012). Interestingly, DNA methylation is reestablished in the vegetative cell via RdDM mediated by small interfering RNAs (Calarco *et al.*, 2012).

Heat stress activates the *ONSEN* (Japanese 'hot spring') retrotransposon and synthesis of extrachromosomal DNA copies in *Arabidopsis* seedlings (Ito *et al.*, 2011). Heat stress triggers the accumulation of *ONSEN* in mutants lacking Pol IV and RDR2, which form the main complex in the RdDM pathway. Interestingly, the memory of heat stress (i.e., transgenerational inheritance of ONSEN insertion) can only occur in the progeny of mutant plants defective in siRNA biogenesis. Moreover, repetitive elements were only found to be active in pollen development (Slotkin *et al.*, 2009). Most of the key genes required for DNA methylation such as *DRM2*, *NRPD1* and *NRPE1* (the largest subunit of plant specific PolIV and PolV) are upregulated during heat stress in *Arabidopsis* (Naydenov *et al.*, 2015), supporting the involvement of DNA methylation under high temperature stress.

Transcriptome studies on heat-treated cotton anthers identified various genes involved in histone modification and DNA methylation. Several genes such as *DNA CYTOSINE-5-METHYLTRANSFERASE* (*DRM1*) and *S-ADENOSYL-L-METHIONINE-DEPENDENT METHYLTRANSFERASE* (*DRM3*) are differentially regulated at different stages of anther development during heat stress, while commonly occurring DNA methylation genes including *NEEDED FOR RDR2-INDEPENDENT DNA METHYLATION* (*NERD*), *NUCLEAR RNA POLYMERASE D1B* (*NRPD1B*), and two *S-ADENOSYL-L-HOMOCYSTEINE HYDROLASE1* (*SAHH1*) were found to be suppressed in the anthers of heat sensitive cotton during heat stress (Min *et al.*, 2014). Studies on DNA methylation dynamics in cotton anthers under heat stress provide strong evidence that epigenetic regulation is required for plants to deal with high temperature stress. Heat sensitive cotton anthers undergo hypomethylation during heat stress.

Furthermore, pollen sterility and defects in anther dehiscence are possibly caused by hypomethylation in heat sensitive cotton (Ma *et al.*, 2018). DNA hypomethylation suppresses expression of genes involved in sugar metabolism and ROS signaling. CHH methylation associated with RdDM showed more prominent changes as compared to CG and CHG methylation, suggesting that heat stress mainly induces the RdDM activity in anthers. Most of the CHH methylation patterns were found in the promoters and downstream regions (Ma *et al.*, 2018). Interestingly, DNA methylation status varies with anther stages. For example, heat tolerant cotton anthers at the tetrad stage have a lower CHH methylation level as compared to heat sensitive cotton under normal conditions. Whereas, at tapetum degradation and anther dehiscence stages, heat tolerant cotton anthers depicted hyper-CHH methylation patterns during heat stress. Hence, heat stress may affect RdDM function in a specific anther stage manner (Ma *et al.*, 2018). These new discoveries shed light on a novel molecular mechanism by which plants ensure the success of male reproduction under high temperature, thus providing new tools for improving crops to adapt to the challenge of global warming. It would be worthwhile to investigate transgenerational epigenetic effects on heat stress tolerance during male reproduction in plants.

3. Abnormal pollen possibly impairs fruit development in strawberry at high temperatures

Heat stress affects pollen formation, which in turn influences fruit development in strawberry. Strawberry fruit weight relies on the number of achenes per fruit (Abbott *et al.*, 1970; Carew *et al.*, 2003; Strik and Proctor, 1988). Achenes produce auxins, which are required for the growth of receptacles during fruit development (Kang *et al.*, 2013; Kronenberg *et al.*, 1959; Nitsch, 1950). Unsuccessful pollination inhibits the development of achenes, resulting in arrested development of the area around unfertilized achenes; thus, strawberry fruits appear small,

misshapen, and partially green. High temperatures (32/27°C and 28/23°C) cause decreased numbers of achenes per fruit of the short-day cultivar Nyoho (Mori, 1998). From appearance of the first inflorescence to anthesis, the pollen viability of Nyoho (a heat insensitive cultivar) was slightly affected by heat stress [30°C/25°C, 2 days]; however, the number of viable pollen of Toyonoka (a short-day heat sensitive cultivar) was significantly reduced (Ledesma and Sugiyama, 2005). In addition, heat stress impaired pollen germination and pollen tube elongation in both cultivars, although Toyonoka showed a higher degree of severity. Under the same heat stress condition, the fruit set of Toyonoka is much lower than Nyoho (Ledesma *et al.*, 2008). Ever-bearing (day-neutral) cultivars Charlotte and Goha grown at 26°C/21°C in a glasshouse produced malformed fruits and further observation revealed that pollen viability and pollen germination rate were reduced (Winardiantika *et al.*, 2014). Collectively, these studies suggest that heat stress causes the formation of abnormal pollen, and the unfertilized achenes subsequently affect fruit development.

Short-term heat stress often occurs during strawberry flowering and fruiting, which is a critical period for strawberry fruit production; however, almost nothing is known about the molecular mechanisms underlying the effects of heat stress on pollen development. The small HSPs in flowers might confer pollen heat resistance for the heat insensitive cultivar Nyoho (Ledesma *et al.*, 2004). During male reproduction, anther cell differentiation, male meiosis, microsporogenesis, and pollen development are highly sensitive to heat stress. To breed heat resistant strawberry cultivars using a combination of traditional and molecular tools, it is imperative to elucidate how strawberry plants respond to heat stress at genetic and epigenetic levels during anther development.

4. Hypotheses and objectives

Elevated temperatures affect fertilization during the summer months, thereby impeding strawberry production in temperate zones. Transcriptomic and proteomic profiling have been widely employed to investigate the molecular mechanism of heat stress using the model plant *Arabidopsis* and major crops, including tomato, wheat etc. Strawberry (*Fragaria sp.*), which is one of the most important fruit crops in the Rosaceae family, is highly sensitive to heat stress during fruit production. So far, it is not clear how the *Fragaria sp.* anther develops and which genes are responsive to high-temperature stress during anther development. Furthermore, although it is well recognized that high temperature causes male sterility, no transcriptomics studies have been performed in *Fragaria* to date. Thus, *Fragaria vesca*, a model system for Rosaceae, provides an excellent model system to elucidate the molecular mechanism underlying heat stress related male sterility, especially for fruit crops.

The goal is to understand the effects of high temperature on plant sexual reproduction at the molecular level and improve the tolerance of crops to heat stress. The main objective of this project is to identify the molecular mechanisms by which heat stress affects anther cell differentiation using the Rosaceae model plant - *Fragaria vesca* (YW5AF7). The central hypothesis is that heat stress impairs anther development by affecting expression of genes responsive to heat stress

In this project, I proposed the following three major objectives to unravel the overall effect of heat on *Fragaria vesca.*

1) Identifying the key stages of *Fragaria vesca* anther development that are most susceptible during heat stress
2) Elucidating underlying molecular mechanisms using a transcriptomics approach
3) Performing functional studies of some key genes identified using RNA-seq, which can be used as putative candidates for thermotolerance

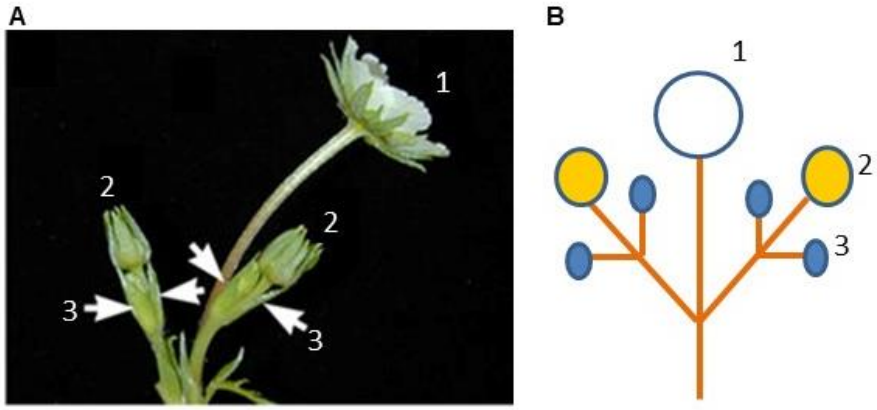

Figure 1.1 *Fragaria vesca* dichasial cyme inflorescence. (A) Primary, secondary, and tertiary flowers in a single inflorescence. Figure (A) from (Hollender *et al.*, 2012). **(B)** Line diagram of inflorescence (denoted with numbers 1, 2, and 3 representing flower positions).

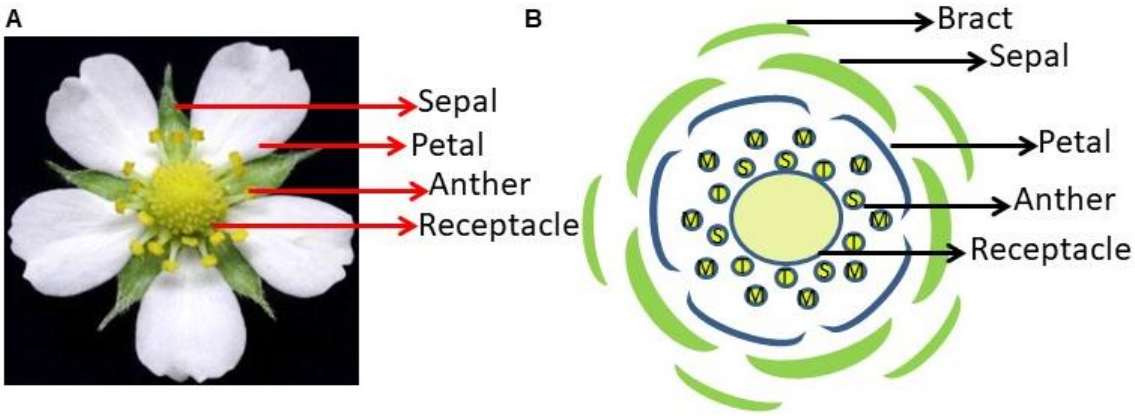

Figure 1.2 ***Fragaria vesca*** **flower morphology. (A)** Open flower representing sepals, petals, anther, and receptacle. **(B)** Floral diagram depicting 5 whorls, starting with bract as the outer most whorl followed by sepals, petals, anthers and receptacle as innermost reproductive whorls.

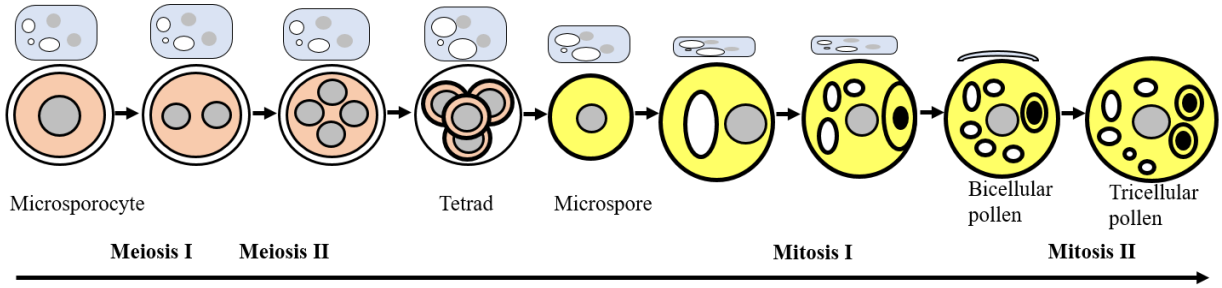

Figure 1.3 Schematic depicting significant events during pollen development.

Tapetal cell (represented with rounded rectangle) and sporogenous cell (rounded).

Table 1.1 Summary of known effects of heat stress on plant male reproductive development

Plant	Temperature	Effects	Reference
Cotton (*Gossypium hirsutum* L.)	35°C to 39°C 7 days	Abnormal microspores and pollen grains	(Min *et al.*, 2014)
Barley (*Hordeum vulgare* L.)	30 °C to 25°C (day/night) 5 days	Abnormal pollen mother cell and tapetum	(Abiko *et al.*, 2005; Sakata *et al.*, 2000)
Rice (*Oryza sativa*)	35-41°C to 15-40°C (day/night)	Anther dehiscence, microsporogenesis	(Jagadish *et al.*, 2007; Satake and Yoshida, 1978)
Tomato (*Lycopersicon esculentum*)	36 °C/26 °C (day/night) 3 days	Affects male gametogenesis	(Giorno *et al.*, 2013)
Triticum aestivum L.	30 °C and 36°C and 26°C (day and night) 3 days	Abnormal sporogenesis and pollen grain viability	(Saini *et al.*, 1984)
Snapbean (*Phaseolus vulgaris* L.)	32.7°C and 32/27°C (1 or 5 days)	Abnormal pollen grains and tapetum	(Gross and Kigel, 1994; Suzuki *et al.*, 2001)
Cowpea (*Vigna unguiculata*)	33°/20° or 33°/ 30°C day/night	Tapetum, tetrads disorganized, Abnormal pollen grains	(Ahmed *et al.*, 1992)
Brachypodium (*Brachypodium distachyon* L.)	36°C	Aborted uninucleate, vacuolated microspore, ruptured tapetal cells, Abnormal pollen grains	(Harsant *et al.*, 2013)

Table 1.2 Summary of some key genes involved in different parts of plant during heat stress response

Model	Temperature	Organs Affected	Gene Involved	Function	Resource
Hordeum vulgare L	30°C five days	MMC and pollen	None	Abnormal pollen grains	(Sakata *et al.*, 2000)
Cotton	35°C to 39°C 7 days	Anthers	*GhCKI, PIF*	Sugar and auxin signaling pathways	(Min *et al.*, 2014)
Maize	41^{o} C for 1 h	Kernels, roots	*HSP101*	Induction of thermotolerance	(Young *et al.*, 2001)
Arabidopsis	44°C for 150 min, 43°C for 25	Seedling	*HsFA1*	Induction of thermotolerance	(Liu and Charng, 2012)
Triticum aestivum	21 to 45 ^{o} C over 1 h	Leaves	*EF-Tu*	chaperone response via chloroplast protein synthesis elongation factor (EF-Tu)	(Fu *et al.*, 2008)
Carrot		Leaves	*shSP17.7*	stabilize membranes by forming heat shock lipids	(Hu *et al.*, 2010)
Arabidopsis	42 or 45 °C 2 hours for 21 days	Seeds	*APX*	seed production	(Suzuki *et al.*, 2013)
Maize, Tobacco		Leaves	*AChE*	heat tolerance upon overexpression	(Yamamoto *et al.*, 2011)
Arabidopsis		Seedling	*PIF*	Signaling	(Leivar and Quail, 2011)

Table 1.3: List of gene networks obtained from transcriptomics studies on anthers of different crops in response to heat stress

Plants	Tissues	Gene network	Conditions	Transcriptomic approach used	Reference
Tomato	Anthers	Heat shock protein, ROS, Sugar and hormone signaling	43–45 °C for 2 h	cDNA-AFLP Affymetrix GeneChip	(Frank *et al.*, 2009)
Tomato	Anthers	Metabolism, transport, heat shock process, oxido-reductive processes	32 °C for 2, 6, 16 or 30 h	Transcriptomics (cDNA-AFLP; 90 K Custom TomatoArray 1.0)	(Bita *et al.*, 2011)
Maize	Anthers	Transcription factors, Starch Biosynthesis, Lipid synthesis	(35°C/25°C light/dark period) for 48 h	Illumina TruSeq Stranded mRNA	(Begcy *et al.*, 2019)
Cotton	Anthers	Sugar and Auxin signaling	35-39°C/29-31°C day/night for 7 days	Illumina HiSeqTM 2000	(Min *et al.*, 2014)
Rice	Anthers	Transcription factors, nucleic acid and protein metabolisms related genes	38 °C for 12 h per day, for 5 days	Illumina sequencing	(Liu *et al.*, 2020)
Rice	Anthers	Metabolic process and related to the catalytic activities	39°C/30°C day/night for 2-4 days	oligo DNA microarray	(Endo *et al.*, 2009)
Barley	Anthers	Heat shock transcription factors, phytohormones related genes,	30°C/25°C day/ night	22K Barley1 GeneChip	(Oshino *et al.*, 2007)
Barley	Anthers	Histone genes and putative lipase gene	30°C/25°C day/ night	SAGE library	(Abiko *et al.*, 2005)

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Chapter 2

Heat stress impairs anther cell differentiation in *Fragaria vesca*

2.1 Abstract

Episodes of heat stress experienced during plant development and reproduction impair male reproductive development. Stress exposure leads to defects or microspore abortion in male reproductive organs, causing male sterility. In an attempt to understand male sterility caused due to high temperature, heat stress (42°C for 2-days and 4-days) was applied to all stages of anther development in *Fragaria vesca*. The results demonstrated that 2-day and 4-day 42°C heat treatments affected anther cell differentiation at as early as stage 6 when meiosis occurs and at post-meiotic stages. However, the severity of 4-day treatment was higher. The development of microsporocytes, tapetum, microspores, and pollen grains is highly vulnerable to heat stress. Moreover, deformed strawberry fruit formation at 42°C was primarily due to reduction in male fertility. Overall, these findings highlight detrimental effects of heat stress on anther cell differentiation.

2.2 Introduction

Elevated temperature as a consequence of climate change affects plant growth, development, reproduction, and yield. An increase in global temperature cause yield loss for major crops such as maize (Lobell *et al.*, 2013), wheat (Begcy *et al.*, 2018), and rice (Begcy and Dresselhaus, 2018; Chen *et al.*, 2016). In particular, plant reproduction is highly sensitive to heat stress. Decrease in crop yield in response to high temperature events mainly results from increased male sterility (Hedhly *et al.*, 2009; Zinn *et al.*, 2010); however, how heat stress exactly causes male

sterility is still not clear. Therefore, it is necessary to identify critical stages during plant male reproduction that are affected by heat stress.

Male reproductive development is vulnerable to temperature fluctuations (Ahmed *et al.*, 1992; Firon *et al.*, 2006; Fragkostefanakis *et al.*, 2016; Giorno *et al.*, 2013; Iwahori, 1965; Kim *et al.*, 2001; Peet *et al.*, 1998). In flowering plants, pollen formation involves a series of complex events taking place inside anther locules, where diploid pollen mother cells undergo meiosis to give rise to four haploid microspores (Goldberg *et al.*, 1993). The released microspores further divide asymmetrically to form a vegetative cell and a generative cell (Ma, 2005; Sanders *et al.*, 1999). During this process of development, the microspore stage is known to be susceptible to temperature fluctuations. In rice, for instance, released microspores were sensitive to low temperatures (Nishiyama, 1984; Oliver *et al.*, 2005) and heat stress causes microspore abortion that leads to male sterility (Begcy and Dresselhaus, 2018; De Storme and Geelen, 2014; Endo *et al.*, 2009; Rieu *et al.*, 2017; Sato *et al.*, 2002).

The tapetum, the innermost layer of the anther locule, is formed along with differentiation of pollen mother cells (Goldberg *et al.*, 1993). At the microspore stage, it provides enzymes, carbohydrates, and nutrients for releasing haploid microspores from tetrads and microspore development. Microspore and tapetum development are well coordinated. For proper pollen development correct timing for tapetum degeneration is essential (Parish *et al.*, 2012). Heat stress causes defects in tapetum at the microspore stage (Ahmed *et al.*, 1992; Suzuki *et al.*, 2001) and irregularities in epidermis, middle layer, and endothecium in anthers (Abiko *et al.*, 2005; Sato *et al.*, 2002).

Apart from microspores, pollen grains are also susceptible to heat stress. For instance, exposure of rice spikelets to high temperature at > 35°C for five days caused pollen sterility and

eventually affected the seed set (Satake and Yoshida, 1978). Reduction in pollen viability also observed in tomato plants grown under mild heat conditions (32 °C/26 °C day/night) (Firon *et al.*, 2006; Pressman *et al.*, 2002). Additionally, pollen grains were deformed and flattened compared to the normal condition (Sato *et al.*, 2002). Pollen production was also affected in *Arabidopsis* flowers corresponding to stage 9 of floral development. Pollen was deformed, where pollen-like structures were observed in anther locules (Kim *et al.*, 2001). One of the plausible causes is premature degeneration of the tapetum that generally leads to aberrant pollen development (Ahmed *et al.*, 1992; Oshino *et al.*, 2007; Parish *et al.*, 2012; Suzuki *et al.*, 2001). In barley and wheat, high temperatures cause male meiosis failure and, consequently, pollen production (Saini and Aspinall, 1982; Sakata *et al.*, 2000). All these episodes of male sterility reveal susceptibility to heat damage at different stages of pollen development. However, it is still not clear how crop plant species respond differently to episodes of heat stress.

Heat stress also impacts fruit formation as a result of abnormal male reproduction. The overall level of fertilization is affected due to lack of sufficient viable pollen, thus forming deformed fruit/unfertilized fruit. For example, heat stress causes male sterility in cowpea by damaging anthers at pollen mother cell stage (Warrag and Hall, 1984). Similarly, in peanut (*Arachis hypogaea* L.), reduction in fruit set occurs at high temperatures when exposed 6 days (Prasad *et al.*, 1999a, b). To maintain proper fertilization and eventually fruit formation, pollen viability during heat stress plays a very important role.

High temperature causes strawberry pollen abortion and consequently fruit deformation. Fruit development in strawberry requires proper fertilization of achenes along with auxin production (Hollender *et al.*, 2014; Kang *et al.*, 2013; Kronenberg *et al.*, 1959; Nitsch, 1950). Unsuccessful pollination of achenes results in distorted fruit formation. Previous reports have

shown a decline in strawberry fruit set as a consequence of poor pollen quality (Gilbert and Breen, 1987). Pollen viability of Toyonoka (a short-day heat-sensitive cultivar) was highly affected by heat stress, which further led to a reduction in fruit set (Ledesma *et al.*, 2008; Ledesma and Sugiyama, 2005). So far, it remains unclear how heat stress causes pollen abortion in strawberry. To address this gap, this study focused on how heat stress causes male sterility in *Fragaria vesca*. The research aimed to examine key anther development stages and identify which are more sensitive to heat stress. Semi-thin sectioning was used to investigate the effect of heat stress on anther development. These studies not only demonstrated the detrimental effects of heat stress on anther cell differentiation, but also paved the way to elucidate the underlying molecular mechanisms in *Fragaria vesca*.

2.3 Materials and Methods

2.3.1 *Fragaria vesca* line and growing conditions

Fragaria vesca "Yellow Wonder" cultivar inbred line generation 7 (YW5AF7)" (Slovin *et al.*, 2009) seeds were sown in moist SUN GRO Metro-Mix 360 (in a 72 cell tray with one seed per pot with 3.5cm (L) X 3.5 cm (B) X4.5cm (H) dimensions each) and stratified at 4°C for 4 weeks. Plants were grown in a growth chamber under a 16-h light/8-h dark photoperiod at 23°C with light intensity of 190 lux and 50% humidity. After appearance of 2-3 trifoliate leaves, seedlings were transferred to individual pots. Plants were watered every two days and fertilized biweekly using Sprint 330 (a chelated iron) and Peterson's Water-Soluble General Purpose 20-20-20 fertilizer that yielded 100-ppm nitrogen and 5 ppm Fe.

For heat treatment, 7-8 plants were subjected to high temperature for 2 and 4 days under a 16-h light/8-h dark photoperiod at 42°C with light intensity of 190 lux and 50% humidity.

Plants in the chamber were checked at regular intervals for water to avoid combined effects of heat and drought stress. After heat treatment, anthers were collected from different bud sizes for further studies.

2.3.2 Pollen staining using Alexander stain

Briefly, flower buds or anthers from control (23°C) and heat-treated (42°C) plants were collected and fixed in Carnoy's solution (ethanol/chloroform/acetic acid, 6:3:1 v/v/v) at room temperature overnight. The following day they were washed with 30%, 50% and 70% EtOH, followed by two washes with autoclaved water. Then the buds were kept for a day in Alexander's stain (95% alcohol, 10 mg Malachite green, 50 ml distilled water, 25 ml glycerol, 5 gm phenol, 5 gm chloral hydrate, 50 mg acid fuchsin, 5 mg Orange G) at 60°C (Alexander, 1969). Anthers were manually dissected from buds and were placed into a drop of distilled water on a microscope slide. Anthers were macerated with a needle prior to being squashed under a coverslip. The slides were observed with an Olympus BX51 microscope equipped with an Olympus DP 70 digital camera. In the Alexander stain, acid fuchsin stains the pollen protoplasm and Malachite green stains cellulose in pollen walls. Pollen grains which stained red were counted as viable, whereas unviable were stained as green shells. The percentage of viable pollen is expressed as the ratio of viable pollen grains to the total grains examined. About 300 pollen grains were counted from two different regions of anthers. Percent viabilities are reported as an average from three replicates of 300 pollen grains each.

2.3.3 Anther semi-thin sectioning and imaging

YW5AF7 flower buds at different stages were collected from 10-15 non-treated (control) and heat-treated plants. Bud size was recorded using a ruler and buds were split in half with the help of a razor blade to allow better infiltration of the primary fixative. After splitting the buds they were immediately placed into glass shell vial containing 5 ml of Trumps 4:1 primary fixative – 4% paraformaldehyde (v/v) (Ted Pella, Product No. 18505 EM grade) and 1% glutaraldehyde (Ted Pella, Product No. 18426 EM grade) (v/v) in 0.1M sodium cacodylate buffer (pH 7.4) and 0.02% Triton X-100 (McDowell and Trump, 1976) overnight followed by three rinses in sodium cacodylate 0.1M pH 7.4 and 0.02% Triton X 100. Tissues were then post-fixed in the secondary fixative- 1% osmium tetroxide diluted from a 4% solution (Ted Pella Co.) overnight at room temperature. After rinsing 2-3 times with 0.1M HEPES buffer, samples were dehydrated using a graded acetone series in 10% increments, up to 100% with duration of an hour each, and finally 100% acetone (Fischer Scientific) thrice each for 60 minutes. Samples were infiltrated with Spurr's resin modified by the addition of Quetol 651 (Adapted from Table 3 of Ellis, 2006, with the exception that 0.05 g 2(dimethyl-amino) ethanol was used in place of 0.20 ml N, N-dimethylbenzylamine (Holdorf *et al.*, 2012). The infiltration was done using 20% increments of modified Spurr's resin diluted in acetone with minimum 3 hours incubation till 100%. One hundred percent resin changes were made 5-6 times over a period of three weeks, using freshly made resin at each change. Individual anthers were dissected out of buds and oriented into flat embedding molds (EM embedding mold 10505-21 cavity, Ted Pella 10505) for cross-sectioning. Anthers in resin were cured at 60 °C for 3 days. Tissue blocks were then block-faced into a trapezium and sectioned using an RMC MT-7 ultramicrotome and glass knives. Semi-thin

sections (0.5 μm) were heat-fixed to slides, stained with toluidine blue (1%) and photographed with an Olympus BX51 microscope equipped with an Olympus DP 70 digital camera.

2.3.4 Scanning electron microscopy backscattered electron imaging

To generate SEM backscattered electron (BSE) images, 400nm sections (from the same blocks used for semi-thin sectioning) were cut, as described above. Sections were carefully placed onto 25 mm round glass coverslips, heated to adhere sections, and the coverslip was mounted onto one-inch aluminum stubs covered with carbon tape. Sections on the coverslip were carbon coated using an Edwards Coating System 306A. After application of carbon paint around the edge of the mounted coverslip that were kept for drying overnight, the anther sections were imaged using an Hitachi S-4800 scanning electron microscope with ExB energy filtering for BSE images (Micheva and Smith, 2007). Images were taken at working distance = 3.3mm using the upper secondary detector with LA15 at 5.0kV voltage and 1.50k magnification.

2.3.5 Visualizing fruit development

Under control and heat-treated conditions, fruit development was tracked starting from the flower being fertilized through mature fruit formation based on previous studies (Hollender *et al.*, 2012). Fruits at a series of developmental stages were photographed using a Canon EOS Rebel T5 camera.

2.2.6 Statistical analysis

Pollen viability data was statistically analyzed using ANOVA in R. Data was considered significant at *$p<0.05$ and ***$p<0.0001$.

2.4 Results

2.4.1 Heat stress leads to deformed fruits

An open flower after 2-4 days after fertilization (DAF) represents the receptacle with swollen achenes. At 6-7 DAF, fertilized achenes cover the whole surface of the receptacle. Moreover, browning of the stigma and style of the carpels are also visible at this developmental stage. At 8-10 DAF, enlargement of the receptacle was observed, whereas at 10-13 DAF well-defined elongated receptacle was formed (Fig. 2.1A).

After 2 days of heat treatment open flowers were marked and tracked for development over the course of time (Fig. 2.1B). No visible differences were observed between control and heat-treated receptacles at 2-4 DAF, but 6-7 to 8-10-DAF receptacles with irregular shapes were observed. The achenes appeared to be unfertilized and hence were not swollen like in the normal developing receptacles. Additionally, the mature accessory fruit was deformed (Fig. 2.1B). Achenes were not formed on the top of the receptacles. Either no or deformed fruits were developed in heat-treated plants.

2.4.2 Heat stress affects pollen viability in *Fragaria vesca*

With Alexander staining, approximately 90.14% of pollen grains were viable under normal conditions (Fig. 2.1A and 2.2D), but the percent of viable pollen grains was reduced to 60.04% after 42°C heat treatment for 2 days (Fig. 2.2B and 2.2D). All pollen grains became unviable after 42°C heat treatment for 4 days (Fig. 2.2C and 2.2D). Thus, the results suggest that heat treatment affects viability of pollen grains.

2.4.3 Normal anther cell differentiation in *Fragaria vesca*

I first characterized anther cell differentiation in *Fragaria vesca* (YW5AF7) grown in control conditions using semi-thin sections. The anther cell lineage follows a significant serial development (Fig. 2.3), dividing the *Fragaria vesca* anther development into early and late anther phases. The early anther development phase comprised the events from initiation of stamen promordia to the completion of meiosis (Fig. 2.4), while pollen development occured in the late phases (Fig. 2.5). Key features including increased sepal lengths in the buds as well as protrusion of the receptacle dome marked the emergence of oval-shaped stamen primordia. The stamen primordia are comprised of three germ layers L1, L2, and L3, the appearance of which is designated as stage-1 (Fig. 2.4G). The developmental fate of these layers is described as follows: L1 gives rise to the anther epidermis, L2 differentiates into archesporial cells, and the L3 layer is destined to give rise to connective tissue (vascular bundles) in later stages (Fig. 2.4H). At stage-3, archesporial cells undergo division to form primary parietal cells (PPC) (Fig. 2.4I). In stage-4 anther, the PPC have given rise to secondary parietal cells in the center of each of the anther lobes (Fig. 2.4J). By stage-5 anther, secondary parietal cells have undergone division to give rise to three somatic layers. From outside to inside they are named as endothecium, middle layer, and tapetum (Fig. 2.4K). Stage-6 of anther development comprises four somatic cell layers, namely the epidermis, endothecium, middle layer and tapetum. These layers surround pollen mother cells (PMCs) surrounded by thick callose walls are well established (Fig. 2.4L). PMCs undergo meiosis at stage-6. All these layers play an important role in later stages of anther development. There is a connection between tapetal cells by plasmodesmata at this stage (Owen and Makaroff, 1995).

After meiosis, the coenocytic pollen mother cells undergo cytokinesis that leads to formation of tetrads at stage-7 anther (Fig. 2.5F). Each tetrad within the anther locules is comprised of 4 haploid microspores held together by a common callose wall. Following this stage, cells of the tapetum releases specific enzymes known as callase (Owen and Makaroff, 1995; Pacini, 1990; Scott *et al.*, 2004). These enzymes dissolve the callose wall, thereby leading to release of microspores from the tetrads in stage-8 anther (Fig. 2.5G).

In late anther stage-10, after the release of microspores, they enlarge and become vacuolated (Fig. 2.5H). The microspores become rounded. Apart from that, enlargement of endothecial cells was observed (Fig. 2.5I and Fig. 2.5J). Lastly, degradation of the tapetum and septum is followed by increase size of endothecial cells and development of endothecial cell wall thickenings. The microspores have undergone mitosis to give rise to pollen grains in stage-11 anther, where remains of middle layer were still observed (Fig. 2.5I). At stage-12, the septum dissolves and the locules enlarge for anther dehiscence. This is the final stage of development, which leads to release of pollen grains (Fig. 2.5J).

In reference to anther stage classification in *Arabidopsis* (Sanders *et al.*, 1999), the major morphological events at each of 13 stages are summarized in Table 2.1. My results showed that the anther cell differentiation in *Fragaria vesca* is essentially similar to that of *Arabidopsis* and my work established the standards for examining how heat stress affects pollen development during anther cell differentiation in *Fragaria vesca*.

2.4.4 Heat stress impairs anther cell differentiation in *Fragaria vesca*

Anthers from buds ranging from small to large sizes were examined for developmental defects during heat stress (Fig. 2.6A-J). After 2 days of heat treatment, the earliest defect was identified

at the meiotic (anther stage-6) stage and abnormalities were also observed at late post-meiotic (anther stage-8) and mature pollen grain (anther stage-12) stages. In the heat-treated anthers at meiotic (anther stage-6) stage, structural differences of epidermis, endothecium and the middle layer were not detected when compared with control anther sections, however the tapetal cells were less stained and abnormally vacuolated (Fig. 2.6F). Additionally, heat stressed plants displayed irregular microsporocytes, while non-stressed plants showed normal anther cell layers along with microsporocytes (Fig. 2.4L).

Strong defects were observed in heat-treated anthers at post-meiotic stages. In stage-8 anthers, the tapetal cells appeared highly vacuolated (Fig. 2.6G). Additionally, microspores had irregular shapes (Fig 2.6G). On the other hand, non-stressed plants displayed normal tapetum and microspores (Fig. 2.5G). Although the elongation of endothelial cells and tapetum degeneration seemed normal in the stage-11 heat-treated anthers, the developing pollen grains were not stained evenly, indicating abnormal pollen vacuolization (Fig. 2.6H). At late stages, shrunken pollen grains, assumed to have been dead at the time of fixation, were aggregated in anther locules, indicating that 2-day heat stress most likely causes pollen death (Fig. 2.6I and Fig. 2.6J), while non-stressed plants presented normal pollen grains inside anther locules (Fig. 2.5J). Moreover, the heat-treated anthers were defective in dehiscence (Fig. 2.6J). These results suggest that heat stress affects tapetal cell and microspore/pollen development. In addition, the harmful effect of heat stress on anther cell differentiation occurred at as early as stage-6 and was intensified at post-meiotic stages.

2.4.5 Prolonged heat stress causes irreversible damage to the developing anther in *Fragaria vesca*

In general, although the impairment was more severe, 4-day treated anthers showed similar defects to those treated for 2 days. At stage-6 anther (meiotic stage), 4-day heat treatment caused vacuolization in both tapetal cells and microsporocytes (Fig. 2.7F). In addition, vacuolated tapetal cells and deformed tetrads were observed in stage-7 anther lobes (Fig 2.7G). With the progression in anther development, heat-treated anthers at stage-8 showed swollen and hypertrophied tapetum cells, which invaded the anther locules, in contrast to stage-7. Moreover, microspores were irregular and appeared severely damaged upon 4 days of heat treatment (Fig 2.7H). Additionally, similar abnormalities were observed in stage-11 anthers where abnormal tapetum along with deformed developing pollen filled the anther locule (Fig. 2.7I). Furthermore, at the late stages in the heat-treated anther locules, there were no pollen grains but only aggregated pollen wall debris (Fig 2.7J). The results suggest that 4 days of heat stress causes hypertrophied tapetal cells along with deformed microsporocyte and abnormal microspores.

2.4.6 Elucidating cellular details by backscattered electron imaging using SEM

To better understand the structural details of anther cell defects caused by heat stress, I examined semi-thin sections using backscattered electron (BSE) imaging by SEM, which can achieve higher magnification and resolution than light microscopy. Because 4 days of heat treatment resulted in extreme damage and sterility, I examined only control anthers and anthers subjected to the effects of the shorter duration (2 days) of heat treatment using this method. Compared with the control, in heat-treated anther lobes at the meiotic stage (stage-6), tapetal cells appeared larger and more vacuolated (Fig. 2.8A and Fig. 2.8B). Moreover, microsporocytes were distorted

after heat stress as compared to the round microsporocytes observed in the non-treated anther lobes (Fig. 2.8A and Fig. 2.8B). Furthermore, irregular callose patterns were observed in heat-treated microsporocytes (Fig. 2.8A and 2.8B).

In control anther lobes at stage-8, when microspores have been released from tetrads, tapetal cell and microspores appeared normal (Fig. 2.8C). Conversely, in the 2-day heat-treated anther lobes tapetal cells contained large vacuoles (Fig. 2.8D). The boundaries of individual tapetal cells were also irregular. Moreover, microspores had irregular shape, lacked cytoplasm, and only remains of the microspore cell walls were observed (Fig. 2.8D). The results show that heat stress damages microsporocytes, tapetal cells, and microspores.

2.5 Discussion

2.5.1 Anther cell differentiation of *Fragaria vesca* is similar to *Arabidopsis*

Unlike the model plant *Arabidopsis*, the process of anther cell differentiation has not been well-established in plants of the Rosaceae family, such as *Fragaria vesca* (Hollender *et al.*, 2012; Sanders *et al.*, 1999). This study classified the anther development series of *Fragaria vesca* into 13 stages, starting from the initiation of stamen primordia till dehiscence of anthers. The progression of anther development in *Fragaria* faces specific challenges due to its determinate dichasial cyme inflorescence. Although significant features of anther development in *Fragaria vesca* have been well defined, this study still lacks some of the developmental stages due to lag in floral bud development. The key features associated with anther cell differentiation in *Fragaria vesca* were similar to that in *Arabidopsis*. The middle layer is much more obvious in *Fragaria vesca* as compared to *Arabidopsis*. In addition, the size of the bud (length) was correlated with the anther development stage. This work provides the basis not only for studying

molecular mechanisms underlying anther cell differentiation, but also for identifying the key stages susceptible to abiotic stress, such as high temperature, in plants in the Rosaceae family, including strawberry.

2.5.2 Tapetum and microspore degeneration leads to abnormal pollen development and male sterility during heat stress

The results show that prolonged heat stress (4 days, 42°C) caused a complete loss of pollen viability, while 60% of pollen grains remained viable under a 2-day short heat treatment. Similar effects of heat stress on pollen viability were observed in various plants such as rice (Endo *et al.*, 2009), cotton (Song *et al.*, 2015), tomato (Pressman *et al.*, 2002), common bean (Gross and Kigel, 1994; Prasad *et al.*, 2002), and cowpea (Warrag and Hall, 1983). Very few studies, however, have been conducted on strawberry plants. Variability in pollen viability was found among different strawberry cultivars during heat stress, e.g., pollen quality and performance from "Nyoho" was less affected than "Toyonoka" at 30/25°C (Ledesma and Sugiyama, 2005). My studies on anther cell differentiation using semi-thin sectioning revealed that heat stress resulted in detrimental effects on male meiosis and development of tapetum, microspores and pollen, along with the progression of anther development. The product of male meiosis followed by cytokinesis, i.e., tetrads, became abnormal upon heat treatment, indicating that heat stress may affect nuclear division or cytokinesis. Heat stress leads to the production of defective microspores. In accordance with these findings, cotton microspores also displayed similar defects upon heat stress exposure for 7 days (Min *et al.*, 2014). Several other crops also exhibit distorted microspores as a consequence of heat stress (Abiko *et al.*, 2005; Endo *et al.*, 2009; Oshino *et al.*, 2007). Moreover, during this study, it was observed that tapetal cells are very

sensitive to high temperature, which has been shown before that heat stress induces formation of vacuolated tapetal cells in other plant species (Abiko *et al.*, 2005; Endo *et al.*, 2009; Oshino *et al.*, 2007; Saini *et al.*, 1984; Suzuki *et al.*, 2001). Thus, this study in strawberry supports findings from other species that microsporocyte, microspore and tapetum are highly susceptible to heat stress.

As a consequence of heat stress, hypertrophied tapetal cells were observed in stage-8 anthers. Similarly heat stress led to large vacuoles in tapetal cells of *Phaseolus vulgaris* L. (Suzuki *et al.*, 2001). Mutants that exhibit hypertrophied tapetum cells fail to produce pollen grains even under normal conditions (Jacobowitz *et al.*, 2019; Millar and Gubler, 2005; Sanders *et al.*, 1999; Sorensen *et al.*, 2003; Wilson *et al.*, 2001; Zhang *et al.*, 2006; Zhang *et al.*, 2007). In later stages of anther development, tapetal cells provide energy and materials for pollen development and pollen coat formation (Huang *et al.*, 2017; Parish and Li, 2010; Wang *et al.*, 2003; Wu *et al.*, 1997). In *Fragaria vesca*, the malfunction of tapetal cells under heat stress might affect the development of tetrads, microspores, and pollen, suggesting an important role of tapetum in response to heat stress.

Unlike light microscopy, SEM backscattered electron (BSE) imaging allows observing structural changes at the sub-cellular level in *Fragaria vesca* anthers. The precise structure of nuclei and vacuoles were observed in the BSE micrographs of semithin sections of *Fragaria vesca* anthers. Analysis by SEM BSE imaging still provided better insights into the structural changes caused by heat stress than examination of semi-thin sections by light microscopy.

The molecular events affected by elevated temperatures remain poorly understood during plant reproduction. Both 2-day (short) and 4-day (prolonged) heat treatments irreversibly impaired tapetum and pollen development in *Fragaria vesca*. The results showed that heat stress-

caused defects in anthers at the meiotic stage (anther stage-6) and became intensified at the post-meiotic stages. Thus, further exploration of effects of heat stress on *Fragaria vesca* anther development at stage-6 (the meiotic stage) and stage-8 (a key post-meiotic stage) will shed light on the molecular mechanisms by which plants respond to heat stress during reproduction.

2.5.3 Male sterility in *Fragaria vesca* leads to formation of abnormal fruits after heat stress

Here I established *Fragaria vesca* as one of the fruit plants that can be an experimental model for high temperature injury. Using this system, it was observed that anthers at stage-6 and 8 of are greatly affected during heat stress. Further, it was observed that male sterility as a consequence of heat stress causes deformed strawberry fruits. Interestingly, similar results were also reported in rice where high temperature affects microspores, leading to reduced spikelet fertility at 39°C/30°C (Endo *et al.*, 2009).

Compared with vegetative growth and development, the reproductive phase in strawberry is more susceptible to high temperatures, which directly reduces the quality and quantity of fruits. Fruit formation depends on successful fertilization; an important criterion for indicating effects of heat stress on plant reproduction is the silique length (fruit for *Arabidopsis*) which corresponds to number of seeds per silique (Bac-Molenaar *et al.*, 2015). In this study, malformed fruits of *Fragaria vesca* display their sensitivity to heat stress. Deformed fruits exhibited unfertilized achenes, which caused irregular appearance, especially at the apex. It is highly possible that poor fruit set is a consequence of defective pollen. Similarly, in the strawberry cultivar “Toyonoka”, heat stress (30/25°C) exposure caused malformed fruits (Ledesma *et al.*, 2008).

I found that heat stress impairs pollen development. It is possible that abnormal pollen caused by heat stress results in poor fertilization and, subsequently, aberrant fruit development. While the process leading to proper development of fruit has been studied (Hollender *et al.*, 2012), little is known about how heat stress directly affects the strawberry fruit development. These results will be beneficial for improving fertilization and fruit production in strawberry under elevated temperatures.

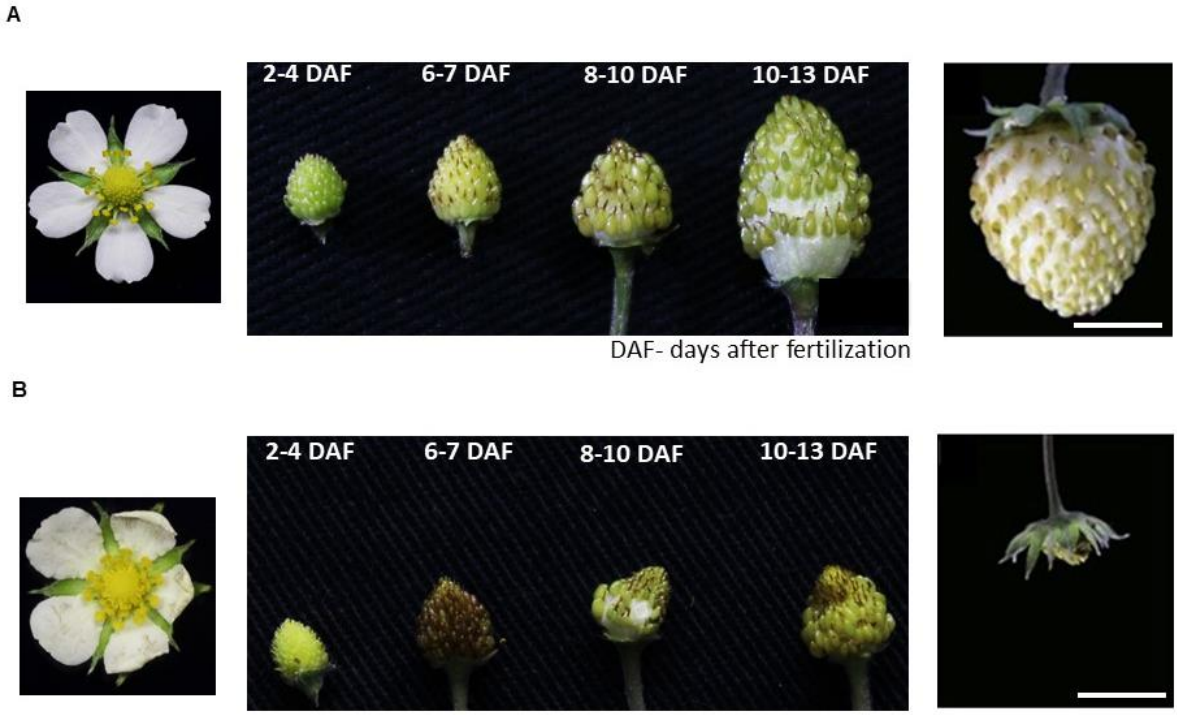

Figure 2.1 High temperature stress leads to deformed fruits in *Fragaria vesca*. (A) Fruit development series under normal conditions (**B)** Fruit development series, when buds were heat treated at 42℃ for 2 days. (DAF-Days After Fertilization). Scale bar = 1cm

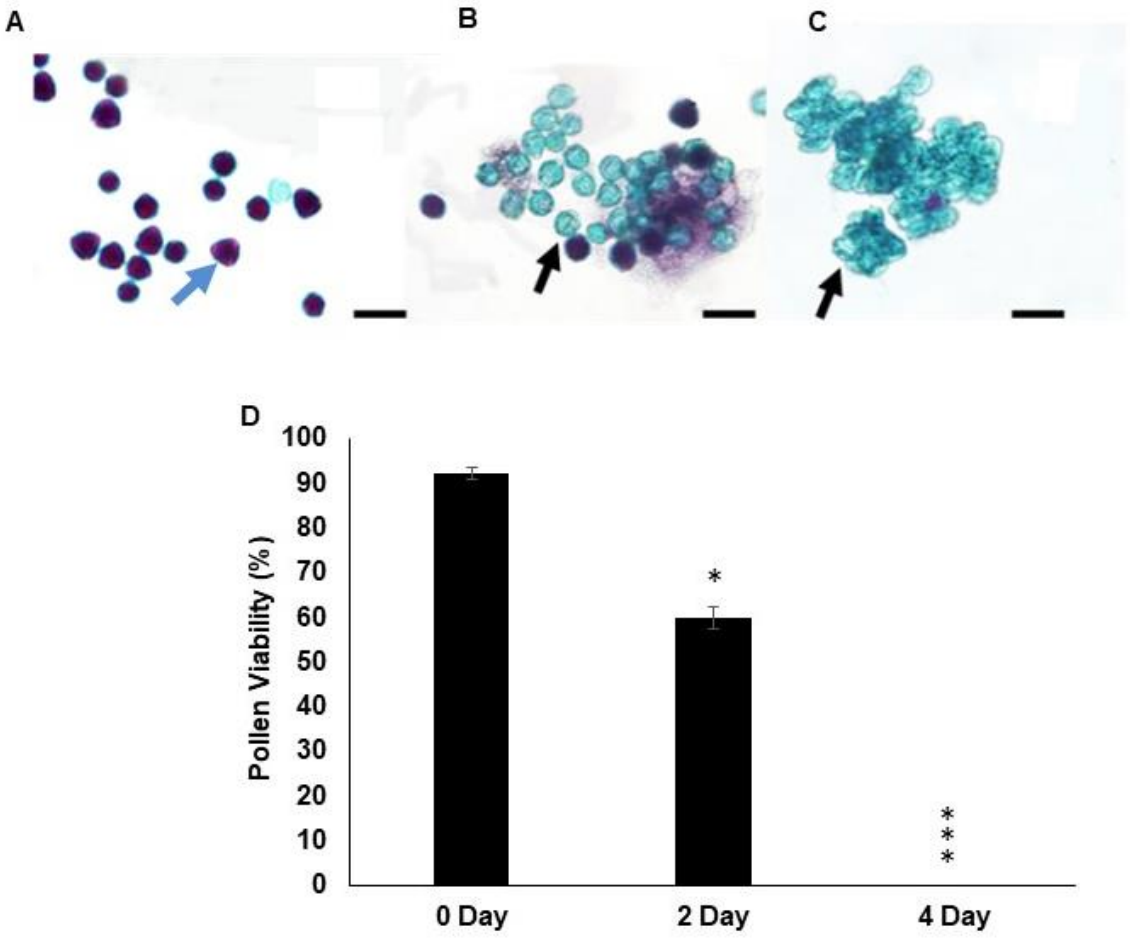

Figure 2.2 Heat stress affects pollen viability. (A) to **(C)** Alexander staining of control and heat-treated pollen grains. **(A)** Pollen grains in control are stained in red (blue arrow), indicating viability. **(B)** Pollen grains after 2-day heat treatment (42°C) are stained in bluish-green (black arrow), indicating unviability. **(C)** All pollen grains after 4-day heat treatment (42°C) are stained only in bluish-green and aggregated (black arrow), indicating no viable pollen. **(D)** Percentages of pollen viability from control and heat-treated anthers. (* $p < 0.05$ and ***$p<0.0001$)

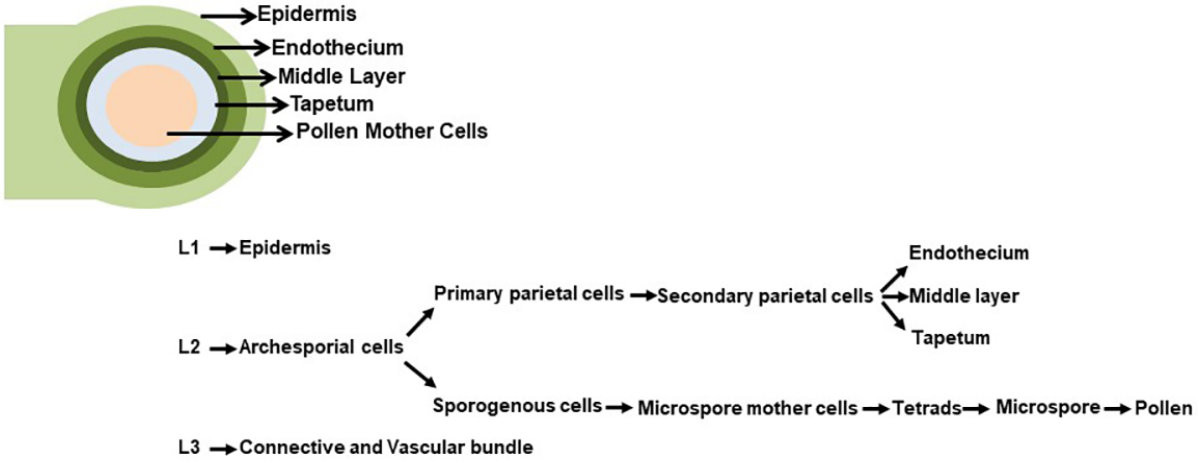

Figure 2.3 Anther cell lineage representing different cell types. An anther lobe representing pollen mother cells and somatic cells (epidermis, endothecium, middle layer, and tapetum). L1 cells form epidermis. L3 gives rise to connective and vascular tissue. L2 differentiates into archesporial cells. They give rise to primary parietal cells and sporogenous cells. Primary parietal cells give rise to secondary parietal cells, which finally result into development of endothecium, middle layer and tapetum. On the other hand, sporogenous cells form pollen mother cells, which undergo meiosis and cytokinesis, giving rise to tetrads. Tetrads eventually release microspores that finally form pollen grains (Ma, 2005; Sanders *et al.*, 1999).

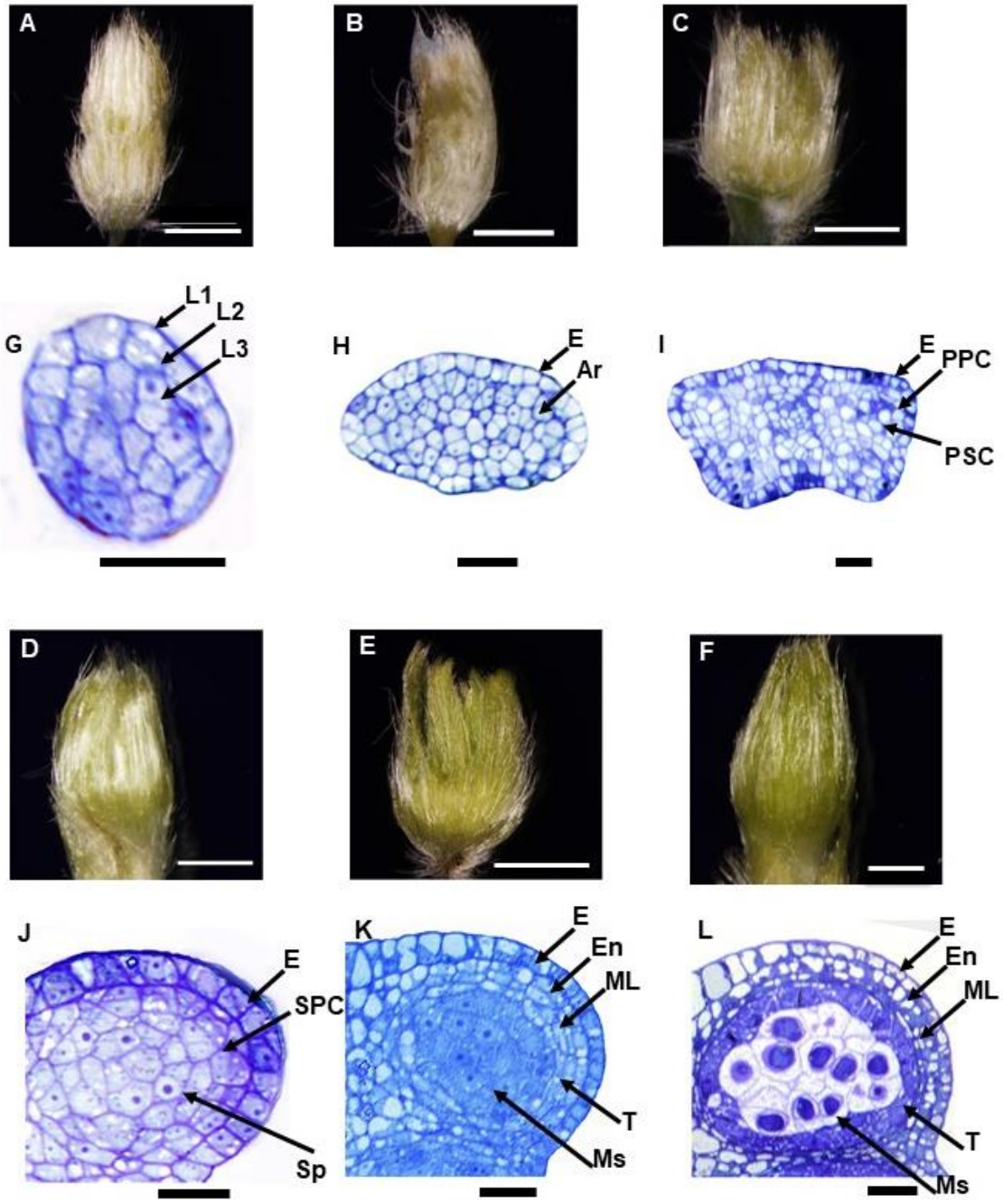

Figure 2.4 Anther cell differentiation in *Fragaria vesca* (semi-thin sections stained with toluidine blue). **Early stages: (A)** to **(F)** Buds from which anthers were sampled for semi-thin sectioning, bars = 1 mm. **(G**) to **(L)** Anther semi-sections, bar = 20 µm. **(G)** A stage-1 anther

showing three layers of cells: L1, L2, and L3. **(H)** A stage-2 anther exhibiting epidermis (E) and archesporial cells (Ar). **(I)** A stage-3 displaying the E, the primary parietal cells (PPC), and primary sporogenous cells (PSC). **(J)** A stage-4 anther lobe showing E, the secondary parietal cells (SPC), and sporogenous cells. **(K)** A stage-5 anther lobe exhibiting microsporocytes (Ms) surrounded by four anther cell layers: E, Endothecium (En), the Middle Layer (ML), and Tapetum (T) thus indicating the completion of anther cell differentiation. **(L)** A stage-6 anther lobe showing E, En, ML, mature T and Ms undergoing meiosis.

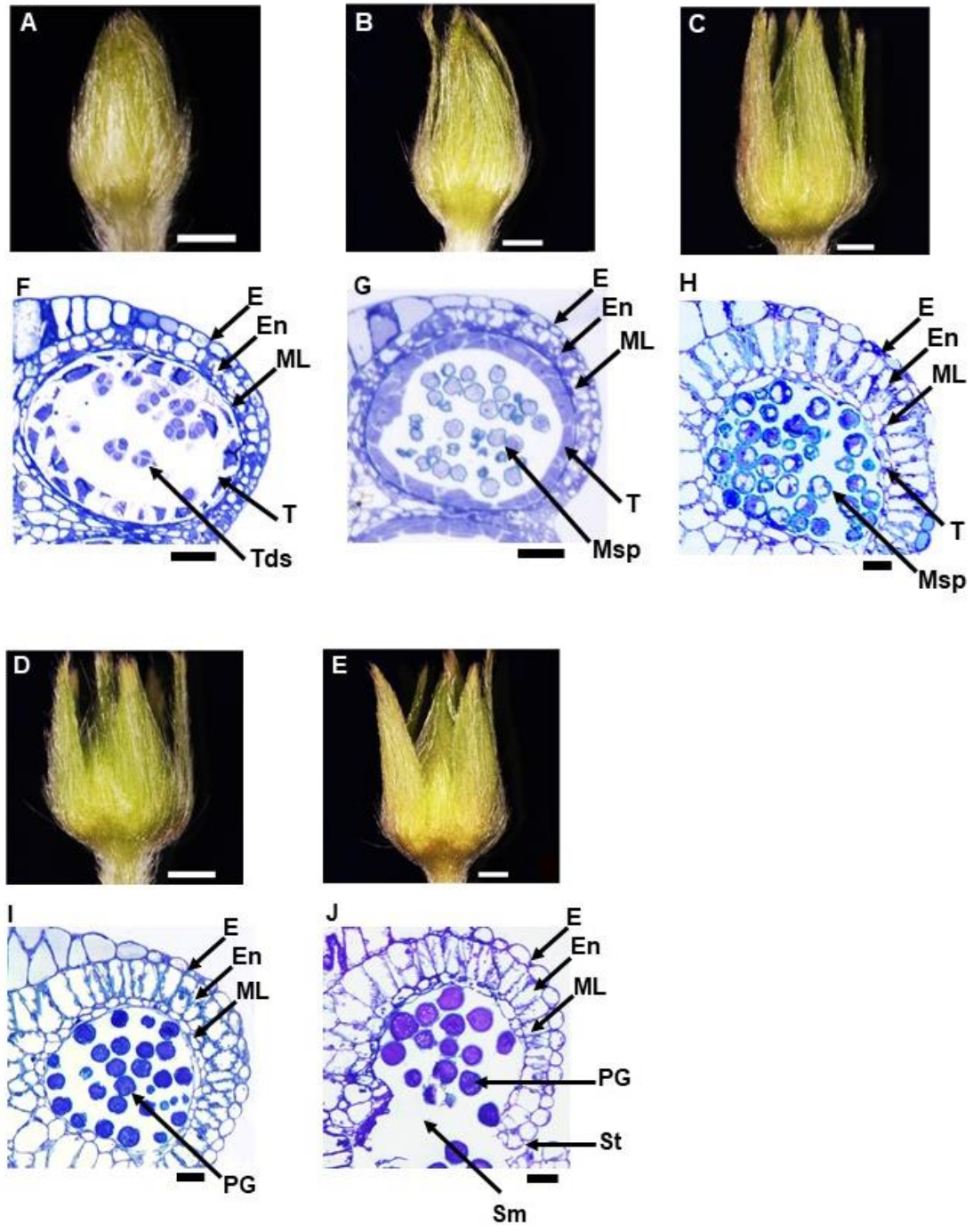

Figure 2.5 Anther cell differentiation in *Fragaria vesca* (semi-thin sections stained with toluidine blue). **Later stages: (A) to (E)** Buds from which anthers were sampled for semithin sectioning, bars = 1 mm. **(F)** to **(J)** Anther semi-thin sections, bar = 20 µm. **(F)** A stage-7 anther

lobe displaying E, enlarged En cells, ML, vacuolated T, and tetrads (Tds), indicating the completion of meiosis. **(G)** A stage-8 anther lobe showing E, enlarged En cells, ML, vacuolated T, and released microspores Msp. **(H)** A stage-10 anther lobe displaying E, elongated En cells, ML, degenerated T, and bicellular pollen grains (PG). **(I)** A stage-11 anther lobe exhibiting E, En cells with thickened cell walls, ML, and mature PGs. **(J)** A stage-12 anther lobe showing PGs releasing from the lobe.

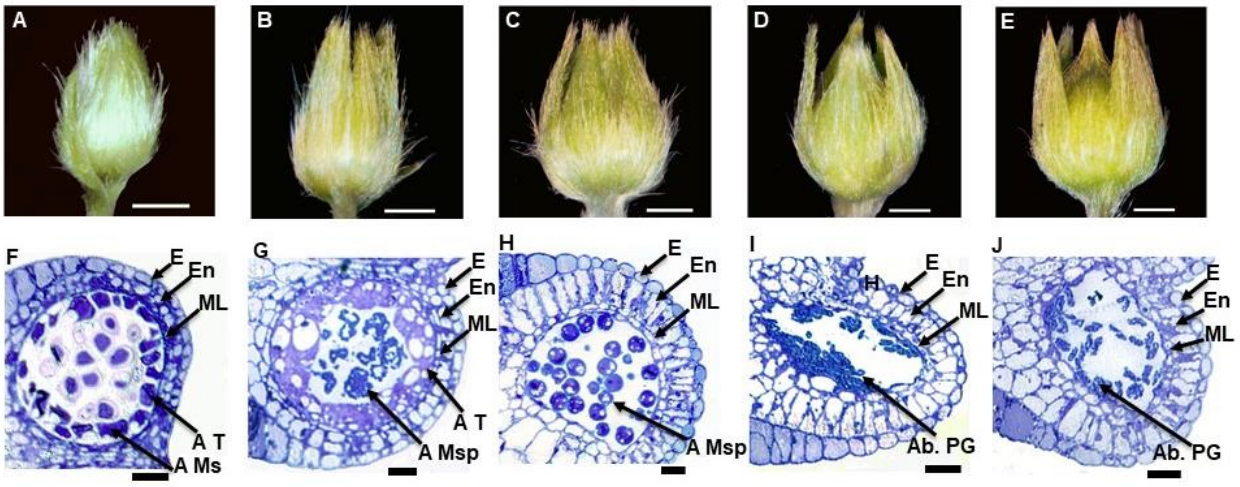

Figure 2.6 High-temperature (42°C for 2 days) causes aberrant anther development in *Fragaria vesca*. (A) to (E) represents the buds (Bar = 1mm) for which anthers were taken for semi-thin sectioning**.** Light microscopy pictures **(F) to (J)** (Bar F=20µm, G-J=50µm) shows sections stained with toluidine blue. Microsporocytes, microspores, tapetum and pollen grains are degenerating after 2 days of heat stress at 42˚C. **(F)** Stage-6, or meiotic stage, with abnormal tapetum (A T) and microsporocytes (A Ms). **(G)** Stage-8 or Post-meiotic stage representing hypertrophied abnormal tapetum (A T) and irregular, deformed microspores (A Msp) in an anther locule. **(H)** Stage-10 displays variability in size of microspores. **(I) to (J)** Represents abnormal pollen (Ab. PG) and remains of dead pollen wall inside the locule.

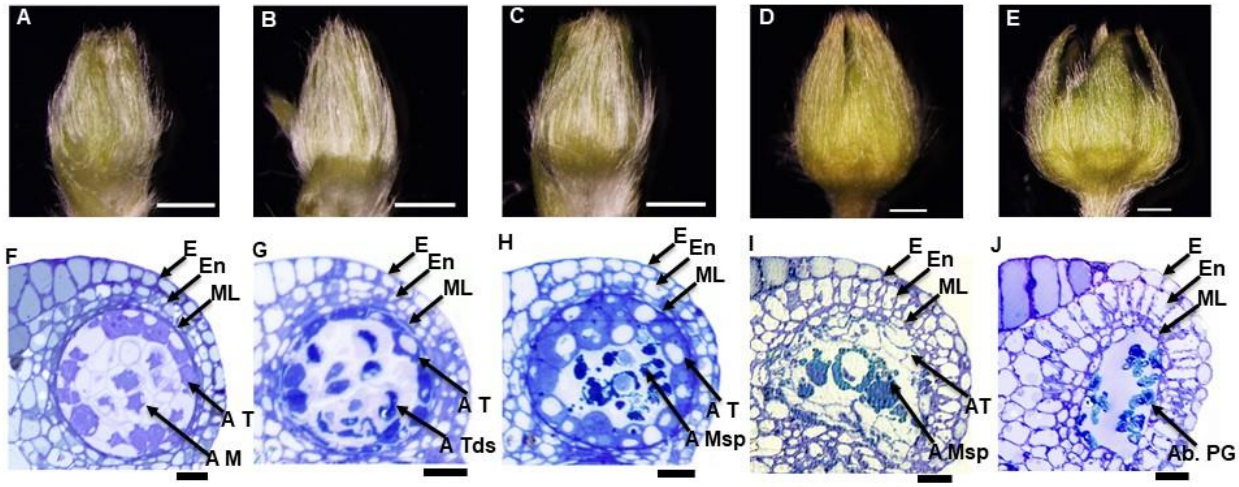

Figure 2.7 Developing anthers of *Fragaria vesca* show aberrant development during prolonged period of high temperature stress. (A) to (E) represents buds (Bar = 1mm) from which anthers were taken for semi-thin sectioning. Light Microscopy pictures **(F) to (J)** (Bar F=20µm, G-J=50µm) show sections stained with toluidine blue. Microsporocytes, microspores, tapetum and pollen grains degenerating after 4 days of heat stress at 42˚C. **(F)** Meiotic stage-6 represents abnormal tapetum (AT) and abnormal microsporocyte (A Ms). **(G)** Stage-7 abnormal tetrads (A Tds) along with defective tapetal cell layer. **(H)** Post-meiotic Stage-8 with hypertrophied abnormal tapetum (AT) and abnormal microspores (A Msp). **(I) to (J)** Representative anther locules with aggregated abnormal pollen grains (Ab. PG)

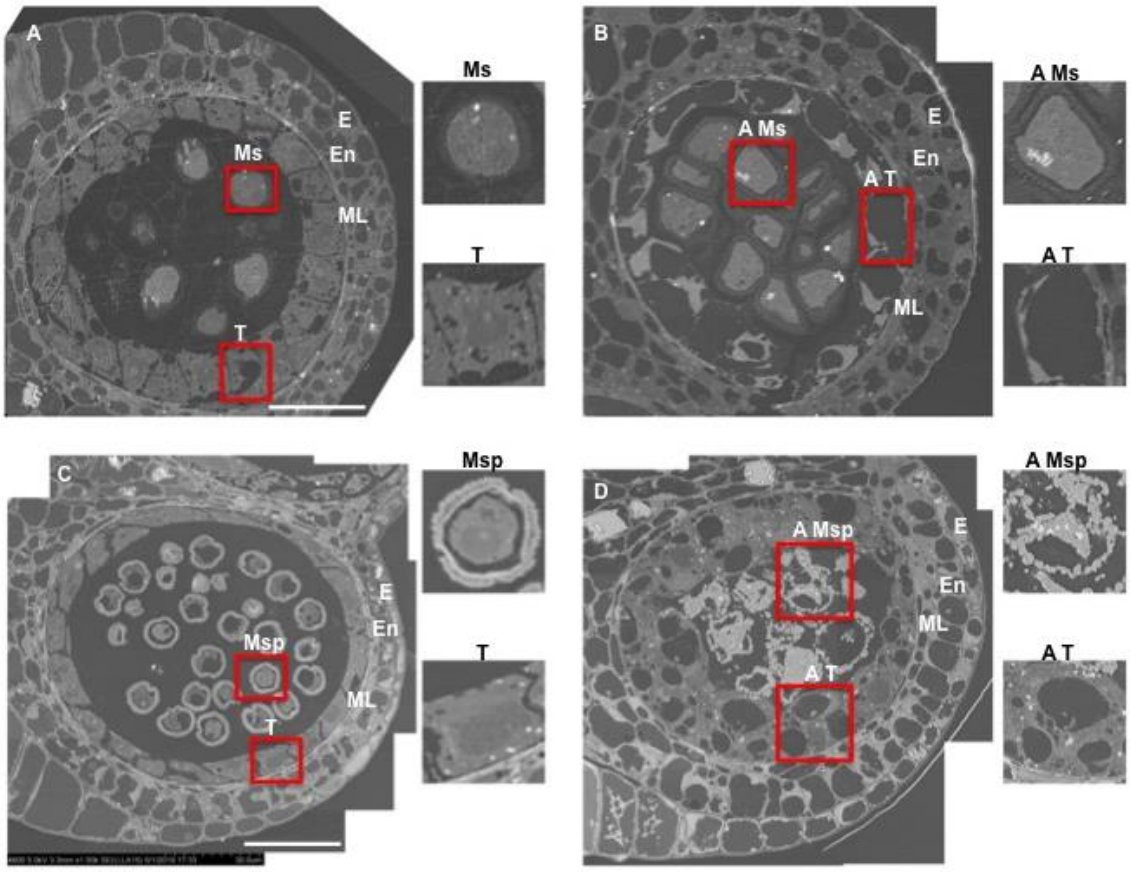

Figure 2.8 Back-scatter SEM micrographs showing anthers during the meiotic stage (stage-6) and post-meiotic stage (stage-8) anthers after 2 days of heat stress treatment: **(A, C)** Non-treated single anther lobe at stage-6 (A) and stage-8 (C) with well-defined anther cell layers. **(B)** 2 days heat-treated single anther lobe (stage-6) showing abnormal tapetum and abnormal microsporocyte surrounded with irregular callose deposition. **(D)** 2 days heat-treated single anther lobe (stage-8) showing with abnormal tapetum representing hypertrophy and irregular boundaries of cell covering most of the locule. Microspores are irregular and were likely dead at the time of fixation surrounded by remnants of cell wall. Individual cell types are outlined with red boxes to indicate the differences. Abbreviations: E- Epidermis, En- Endothecium, ML-

Middle Layer, Ms- Microsporocyte, Msp- Microspores, AT- Abnormal Tapetum, A Ms-Abnormal Microsporocyte, A Msp- Abnormal Microspores. Scale bar 30µm

Table 2.1: Summary of major events during *Fragaria vesca* anther development

Bud size (mm)	Anther stage	Major morphological events
0.4 to 1.0	1	Round stamen primordia with emergence of L1, L2 and L3 cell layers
	2	Oval shaped primordia with distinct epidermis and archesporial cells derived from the L2 layer
	3	Appearance of primary parietal cells and primary sporogenous cells from archesporial cells
	4	Formation of four-lobed anther structure with secondary parietal cells and sporogenous cells
1.5 to 3.0	5	All anther cell types present, pollen mother cells surrounded with epidermis, endothecium, the middle layer, and tapetum
	6	Occurrence of meiosis in pollen mother cells
	7	Pollen mother cells complete cytokinesis, resulting in formation of tetrads
3.5 to 4.5	8	Callose wall surrounding tetrads degenerates, causing the release of individual microspores
	10	Microspores become vacuolated, tapetum and the middle layer still present, endothelial cell elongates, and cell wall thickens
	11	Pollen undergoes mitosis, no remains of tapetum, stomium differentiation takes place
5.0 to 6.0	12	Anther becomes bilocular and contains tricellular pollen grains, septum disintegrates
	13	Dehiscence and pollen release

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Chapter 3

Stage-specific transcriptomics of heat stressed anthers reveal transcriptomic alterations in *Fragaria vesca*

3.1 Abstract

Development of male reproductive tissues is known to be highly susceptible and sensitive to heat stress. Exposure to heat stress leads to abnormal anther development and male sterility. However, little is known about changes in gene expression when anthers are exposed to heat stress. To elucidate the molecular mechanisms mediating heat stress effects on anther development, RNA-seq analysis was performed on *Fragaria vesca* anthers under normal conditions and heat stress at 42°C for 2 days at meiotic (stage-6) and post-meiotic (stage-8) stages. Heat stress-responsive genes including those encoding heat shock factors and proteins, were highly expressed during heat stress. Moreover, relative to controls, expression of genes associated with histone modifications, cell wall formation, oxidation-reduction, and transcriptional regulation were induced by heat stress. In particular, expression of *HEAT SHOCK PROTEIN 70*, *HEAT SHOCK PROTEIN 90*, and *HISTONE DEACETYLASE 6* dramatically increased in both anther stages upon heat stress. The meiotic stage-6 appears to be more active in gene transcription than the post-meiotic stage of anther development. This study identified critical heat response genes that may play important roles in mitigating the effect of heat stress during anther development in *Fragaria vesca*.

3.2 Introduction

High temperatures are known to cause damage to plants. Average global temperature has increased by 0.13°C per decade since 1950 (Solomon *et al.*, 2007) and the daily maximum

temperature will increase by ~1 to 3°C by mid-21st century (Field *et al.*, 2012). The rise in temperature has led to decrease in plant yield with major crops affected including maize, rice, cotton, and tomato (Begcy *et al.*, 2019; Ding *et al.*, 2017; Min *et al.*, 2014). Heat stress also affects fruit crops by decreasing their overall quality and shelf life. To better predict crop responses to global warming conditions, more detailed understanding of the responses of plants; especially reproductive development to heat stress is needed.

Several crops are most affected at the male reproductive stage during high-temperature waves. In flowering plants, male reproductive development undergoes a series of complex events to give rise to pollen grains (Goldberg *et al.*, 1993; Ma *et al.*, 2012). This involves the formation of the microsporocyte, also referred to as the pollen mother cell, which undergoes meiosis I and II, followed by cytokinesis, producing microspores. Each microspore then undergoes mitotic division to produce a vegetative cell and a generative cell. Several reports indicate different stages of male reproductive development that are most vulnerable during heat stress, including microspores and pollen grain stage (Endo *et al.*, 2009; Fragkostefanakis *et al.*, 2016). Interestingly, studies performed on various anther development stages in various crops at the transcriptional level to identify the key genes involved during heat stress. For instance, transcriptome analysis of heat-stressed tomato microspore revealed heat shock factors and proteins, oxidative stress response genes, sugar metabolism genes, and ethylene genes in response to heat stress (Frank *et al.*, 2009). Moreover, carbohydrate metabolism was highly affected in cotton anthers during heat stress. Most of the genes expressed in cotton anthers encoded for enzymes involved in starch and sucrose metabolism (Min *et al.*, 2014a). These studies indicate that heat stress affects various aspects of molecular signaling in plants.

Generally, strawberries grow in temperate regions, which, when affected by intermittent heat waves, make the fruit vulnerable to heat stress. The fruit formation of strawberry is highly dependent on the fertilization of the carpels on the receptacle (Nitsch, 1950), and all this is well correlated with the proper function of the male reproductive system. The effect of heat stress on male reproductive development is still unexplored. There are no reports about how heat stress causes male sterility and its underlying regulatory mechanisms.

To characterize key genetic processes involved in male reproductive developmental responses to heat stress, *Fragaria vesca* was examined as a representative of the Rosaceae family. In Chapter 2 of this dissertation, two critical stages of anther development were identified as highly affected during high-temperature stress, but the gene networks involved in this sensitivity of anther development in *Fragaria vesca* during heat stress are still unknown. This study examines critical genes and pathways essential during anther development in response to heat stress. RNA-seq was used to investigate the effect of heat stress on the meiotic (anther stage-6) stage and the post-meiotic (anther stage-8) stage of anther development and identify the genes involved in responses.

3.3 Materials and Methods

3.3.1 RNA Methods

3.3.1.1 RNA Extraction

Anthers from control and heat-treated (2-days) *Fragaria vesca* buds at anther stage-6 (meiotic) and anther stage-8 (post-meiotic) were collected for total RNA preparation. For collecting specific anther stages, anthers from the buds were hand dissected. They were first checked for correct stage by gently squashing open one of the anthers from each bud using fine needles on a

microscope slide, followed by observation with a compound microscope. When a bud was determined to be at a correct stage, the remaining anthers left inside the bud were collected using a dissection microscope and preserved in RNAlater™ solution (Sigma-Aldrich, St. Louis, MO, USA), an RNA stabilization reagent. My results show that 300-350 anthers at the post-meiotic stage (anther stage-8) and 400 anthers at the meiotic (anther stage-6) stage are sufficient for extracting high quality of total RNA. The samples were ground using DEPC-treated pestles, and the ground samples were then used for RNA extraction. RNA was isolated using the RNeasy plant mini kit (Qiagen) following the manufacturer's protocol. Three independent biological replicates were used. The eluted RNA was treated with DNase (NEB) following the manufacturer's protocol, which was further evaluated by agarose gel electrophoresis of a subsample of each RNA, and quantification using a Qubit 3.0 Fluorimeter (Fisher Scientific).

3.3.2 RNA-seq Library Preparation

RNA-seq libraries were prepared using TruSeq Stranded Total RNA Sample Preparation (Low Sample Protocol) (Illumina, San Diego, CA). I followed the procedure described below to construct RNA-seq libraries.

1. mRNA enrichment: RNA was diluted using nuclease free ultra-pure water in a new 96-well plate (0.3ml PCR) labeled with RBP (RNA bead plate) barcode. RNA purification beads were added to bind the polyA RNA to oligo-dT beads. This was followed by mRNA denaturation in a thermal cycler, thereby facilitating binding of the polyA RNA to the beads upon incubation at room temperature. The plate was then placed on a magnetic stand to separate polyA RNA bound beads. After removal of the supernatant, beads were washed with washing buffer. This step aids in rRNA depletion thereby indicating a strong signal for an RNA population of interest. Then

elution buffer was added to elute mRNA from the beads (using a thermal cycler). After this, bead-binding buffer was added to each well of RBP plate thereby allowing mRNA to specifically rebind the beads.

2. RNA fragmentation: After purification, the remaining mRNA was fragmented into about 100-300 base pair long strands via RNA hydrolysis. Further, beads were washed with washing buffer. Then elute, Prime, Fragment Mix was added which serves as the first strand cDNA synthesis reaction buffer and also contains random hexamers for RT priming. The plate was placed on thermal cycler to elute, fragment, and prime the RNA.
3. Synthesis of double stranded cDNA: This was immediately followed by first strand cDNA synthesis involving fragmented and primed mRNA, First Strand Master Mix tube and reverse transcriptase using thermal cycler. After synthesis of the first strand cDNA, Second Strand Master Mix was added for synthesis of a replacement strand to generate ds cDNA. For separation of ds cDNA from the second strand, the reaction mix AMPure XP beads were used. The supernatant was removed, and beads were washed with 80% EtOH several times. Beads were allowed to dry at room temperature and then resuspended in the Resuspension Buffer. The plate was kept on magnetic stand and supernatant containing the ds cDNA was transferred to a new plate. End Repair Mix was added to the plate containing ds cDNA, using thermal cycler to convert overhangs resulting from fragmentation into blunt ends. This was followed by the addition of AMPure XP beads and washing with ethanol as a clean-up step.
4. Adapters ligation: A-Tailing Mix was added to add a single ‘A’ nucleotide to the 3’ ends of the blunt fragments, hence, preventing self-ligation during adapter ligation reaction. A corresponding single ‘T’ nucleotide on the 3’ end of the adapter provides a complementary overhang for ligating the adapter to the fragment. After adenylation, Ligation Mix along with

RNA Adapter Index was added to each well of the plate and incubated using a thermal cycler. To inactivate the ligation, Stop Ligation Buffer was added. The cleanup step involved AMPure XP beads, followed by several ethanol washes. After the beads were air dried, they were suspended in Resuspension Buffer. Again, second and third cleanup was performed using the supernatant and adding AMPure XP beads, washing with ethanol and then re-suspending in Resuspension buffer.

5. PCR amplification: Finally, PCR Primer Cocktail and PCR Master Mix were added, and PCR was run to selectively amplify DNA fragments having adapter molecules on both ends. PCR cleanup being the last step before library validation was conducted via adding AMPure XP beads to PCR amplified library followed by two ethanol washes, air drying pellet and then resuspending in Resuspension Buffer.

The libraries were then sent to UW-Madison Biotechnology Center for validation, pooling, sequencing on Illumina HiSeq 2500 to acquire 100 base pair, single end reads.

3.3.3 RNA-seq Data Analysis

For this study, I made 12 RNA-seq libraries using anthers from two stages (meiotic and post-meiotic) of buds from both control and 2-day heat-treated plants, with three replicates each. The in-depth outcompeting transcriptomics study involving Illumina HiSeq2500 RNA-seq lead to 17 and 40 million bp single end reads from each of the three replicated libraries. To profile the differentially expressed genes for the two chosen anther stages, I first trimmed the adapters for each library using the Trimmomatic tool [Michael Thon (2016) Trimmomatic SE- https://de.cyverse.org/de/?type=apps&app-id=e45dcdda-bf73-11e5-98ea-cbc79a6c2167&system-id=de]. Then the trimmed libraries were aligned against *Fragaria vesca*

genome ftp://ftp.bioinfo.wsu.edu/species/Fragaria_vesca/Fvesca-genome.v4.0.a1/assembly/ using HISAT2 (Kim *et al.*, 2015a), a general purpose read aligner for RNA sequencing. It helps in finding target regions in the reference genome that have the largest overlap with them, thereby performing a local alignment for reads. This further helped in using these reads to be assigned to genes for expression analysis. The next step involved assigning mapped reads or fragments to genomic features such as genes, exons and promoters using HTSeq. It is a program for counting RNA-seq reads for genomic features. The input files will involve i) aligned reads in SAM/BAM format and ii) genomic features file in Gene Transfer Format (GTF) source: Fragaria_vesca_v4.0.a1.transcripts.gff3.gz. The output obtained is in the form of a count table that indicates the number of reads assigned to each feature in each library. The next step involved finding the differentially-expressed genes using the reads obtained from HTSeq counts which were used for DEseq2 (Anders and Huber, 2010). This gave output upon comparison of the control (anther stages meiotic and post-meiotic) sequences with the heat-treated (anther stages meiotic and post-meiotic) sequences. The result gave estimated log2fold changes and *p* values for the differentially expressed genes (DEGs) for the two key stages. For meiotic stage, P value <0.05 was used, whereas for post-meiotic stage $P<0.05$ along with alpha value of 0.5 was used to obtain a list of DEGs. Furthermore, as the original GTF file does not have gene names (annotations) for the gene ID obtained, the names were manually searched for in ftp://ftp.bioinfo.wsu.edu/species/Fragaria_vesca/Fvesca-genome.v4.0.a1/functional/.

3.3.4 Functional Annotation of genes

To identify genes and gene networks that are involved in heat stress during anther development, I used GO (Gene Ontology) agriGO v2.0 (Du *et al.*, 2010; Tian *et al.*, 2017) and REVIGO (Supek

et al., 2011) platforms to analyze functions of DEGs and hence concatenate them into various cellular pathways. The identification of differentially active pathways and possibly the connection of molecular information (transcriptome) with the phenotype of anther cells helped in elucidating novel interactions in pathways during heat treatment at the two different stages of anther development in *F. vesca*.

3.3.5 Statistical Analysis

For statistical analysis, Principal Component Analysis (PCA) was used to confirm the relationship between libraries.

3.3.6 Heat maps

Heat maps were constructed in R using Pheatmap function. Individual genes for different categories were searched manually from both the meiotic and post-meiotic differential gene lists.

3.4 Results

3.4.1 Variability within the transcriptomic data: Principal component analysis

The difference between treatments is visualized in principal components analysis (PCA) (Fig. 3.1). A total of 90% variation in libraries was observed for the first two principal components (PC1 and PC2). PCA explained the variability in heat-treated and control stages of the anther. The control early and later libraries (TCS5.1, TC5.2, TC5.3, TC10.1, TC10.2, and TC10.3) clustered together, whereas the heat-treated early and late libraries (TH5.1, TH5.2, TH5.3, TH10.1, TH10.2, and TH10.3) were clustered separately (Fig. 3.1). The distance between heat-treated and control samples shows clear differences between these treatments.

3.4.2 Stage specific transcript profiling of *Fragaria vesca* anthers

A total of 4811 differentially expressed genes were detected in the meiotic stage (anther stage-6), and 3553 differentially expressed genes in post-meiotic (anther stage-8) stages after log fold 2 change was used as a cut off value. The result shows an increased number of genes in meiotic stage as, ideally, it represents the early stage of anther cell differentiation with well-defined layers and pollen mother cells (microsporocytes). A total of 2735 genes were up-regulated transcriptionally in meiotic stage (anther stage-6) compared to 951 in the post-meiotic stage (anther stage-8). In contrast, about 675 genes were found to be common for both stages. Additionally, 1647 genes were down-regulated in post-meiotic stages and 1121 in meiotic with about 280 common genes under both stages (Fig. 3.2). Gene Ontology (GO) enrichment analysis of meiotic anther stage-6 revealed oxidation-reduction process, histone-lysine methylation, 7-methylguanosine mRNA capping, transcriptional regulation, response to salicylic acid, cell-wall biogenesis, and photosynthesis as the main categories enriched in post-meiotic anther stage-8 during heat stress (Fig. 3.3). Whereas in the case of post-meiotic anther stage-8 enriched gene categories involved oxidation-reduction process, cell wall organization and metabolism, photosynthesis, and regulation of transcription (Fig. 3.4).

3.4.3 Heat stress responsive genes are differentially expressed in meiotic and post-meiotic stages

A search for "heat" term in the differentially expressed gene list yielded genes associated with conventional heat stress response in both meiotic and post-meiotic stages. Most of them were related to heat shock proteins. A higher number of genes were up-regulated in meiotic stage than post-meiotic (Fig. 3.5; Table 3.1). RNA-seq data analysis revealed some of the common highly expressed genes in both stages include *FvH4_2g04440*, FvH4*_2g01900*, and *FvH4_2g04850*

representing heat shock protein 70 (Table 3.1 and Table 3.2). Moreover, data indicated increased expression of a common gene under the heat shock protein 90 category, i.e., *FvH4_2g13170*, in both stages of the anther. In addition to HSPs, I observed a common heat shock factor (HSF)-type, DNA binding gene (*FvH4_6g17890*), which was up-regulated due to heat stress. Apart from the genes mentioned above, heat shock protein DnaJ, cysteine-rich domain *FvH4_4g22760,* was also up-regulated in response to heat stress. Although no specific trend was obtained, most of them were confined to hsp70 and hsp90 families. Most heat stress-responsive genes found were preferentially expressed in the meiotic stage (Table 3.1).

3.4.4 Histone modification genes are differentially expressed in response to heat stress

A search for "histone" term in the differentially expressed genes yielded genes associated with histone modification. Histone modification genes were highly regulated due to heat stress in both anther stages. Histone constitution genes such as *HISTONE 2A* and *2B* were down-regulated in the case of post-meiotic anther stage-8. In the case of meiotic anthers, a similar trend was observed including *HISTONE 2A*, *2B* and *H4* genes (Table 3.3 and Table 3.4). The data set also shows higher expression of *FvH4_1g25790* in both meiotic and post-meiotic anther stages (anther stages-6 and 8, respectively) (Table 3.3 and Table 3.4). This gene corresponds to its homologue *HDA6* in *Arabidopsis*. Another histone modification gene belonging to deacetylase family i.e., *FvH4_3g26570* has higher expression during heat stress in meiotic anthers, corresponds to *HDA8* in *Arabidopsis* (Table 3.3). Following this trend next is *FvH4_3g41380*, which was also highly up-regulated in the meiotic anther stage, whereas it was not expressed in the post-meiotic anther stage. A heat map of all the genes represents a clear distinction of differential regulation between meiotic stage-6 and post-meiotic stage-8 anthers (Fig. 3.6).

Furthermore, histone modification genes' expression reveals the meiotic anther stage to be more active in transcriptional regulation during heat stress (Fig. 3.6; Table 3.3 and Table 3.4).

3.4.5 Expression of cell wall genes is highly altered in meiotic and post-meiotic anther stages

agriGO analysis demonstrated enrichment cell wall genes in both stages of anther development during heat stress. One of the most enriched GO terms in heat-treated stage-6 and 8 anthers was GO:0005618 belonging to the cell wall category (Fig. 3.7 and Fig. 3.8). A subset of genes enriched under the cell wall category involved glycoside hydrolase and pectinesterase catalytic domain (Table 3.5 and Table 3.6). Overall meiotic stage-6 anther displayed higher glycoside hydrolase 16 f gene expression compared to post-meiotic stage-8 anther. Whereas *FvH4_4g09230*, *FvH4_4g09250*, *FvH4_6g38190*, *FvH4_6g38150*, and *FvH4_4g09160* genes belonging to the glycoside hydrolase 16 family displayed higher expression in both stages of *Fragaria vesca* anther (Table 3.5 and Table 3.6). The data set also showed higher expression of pectinesterase *FvH4_6g17430* gene in anther stage-6 and stage-8. The pattern of differentially expressed genes belonging to pectinesterase was not consistent. Whereas the expression of another pectinesterase gene, *FvH4_1g06350*, was highly reduced in both stages (Table 3.5 and Table 3.6).

3.5 Discussion

3.5.1 Heat transcriptomics data reflects differences in control and heat-treated anthers

This study demonstrates meiotic anther development to be highly active and sensitive to heat stress. The number of differentially expressed genes with heat stress was much higher in meiotic stage-6 than to the post-meiotic stage-8 of anther development. The genes were within a variety

of categories including transcription factors, heat responsive genes, and cell wall associated genes. Previously, different gene regulatory networks associated with different stages of anther development were reported in *Fragaria vesca* (Hollender *et al.*, 2014). Some of the highly expressed gene categories in microspore mother cells undergoing meiosis involve protein degradation and FBX genes, whereas in the case of stage-10 and 11 anthers FBX genes were down regulated. Previous reports also indicate the enrichment of metabolic processes in normal *Fragaria vesca* anthers (Hollender *et al.*, 2014). In contrast, some of the highly expressed genes were found to be Transcription factor GaMYB, Adiponectin Receptor (ADIPOR)-like and Cyclic-Nucleotide-Gated (CNG) channel α3. With MapMan bin enrichment, protein degradation category was obtained in case of stage-9 anthers whereas for pollen categories under transcription and cell wall were enriched the most (Hollender *et al.*, 2014). The presence of cell wall associated genes enriched in both my data and normal anther development data supports the morphological changes obtained during those development phases. The transcriptomics data further diverges from control anther development where heat-induced genes were not reported.

3.5.2 Heat responsive genes play a vital role during heat stress in anthers

Transcriptomics data revealed heat shock protein 70 and 90 highly expressed in heat-treated anther stage-6 and stage-8 (Table 3.1 and Table 3.2), suggesting the importance of heat shock proteins in male reproductive tissues. Likewise, tetrad stage of maize anther also displayed heat shock proteins as one of the hub gene clusters, where *HSP70* (GRMZM2G024718) was highly upregulated (Begcy *et al.*, 2019). Heat stress response causes higher expression of heat shock proteins in reproductive tissues. Heat stress responsive genes such as heat shock factors and heat shock proteins have been widely reported in plants. During normal conditions activation of heat

stress genes is usually associated with developmental functions (Mascarenhas and Crone, 1996). But they play an important role during heat stress periods to protect the tissues at the molecular level. The current transcriptomics data of meiotic anther stage of *Fragaria vesca* also exhibited up-regulation of *FvH4_2g01420, FvH4_2g01780* and *FvH4_2g02500* genes belonging to heat shock protein 70, indicating that early stage of anther development is more active in response to heat than post-meiotic anther stage. *HSP70* significantly increased in tomato microspores during heat stress. Similarly, involvement of *HSP90* was also observed during heat stress response (Frank *et al.*, 2009). In case of rice anthers, expression of heat stress protein genes was also observed to be upregulated during heat stress. Some of the heat-induced genes include ClpB1, DnaJ domain-containing genes along with *LOC_Os04g01740* (HSP90), *LOC_Os03g14180* (HSP20), and *LOC_Os05g35400* (HSP 70) (Liu *et al.*, 2020). These results suggest that male reproductive tissues exhibit a conserved mechanism in response to heat stress by inducing HSP70 and HSP90.

The differential gene expression data also revealed induced expression of *FvH4_6g17890* (*FvHSFA3*), a heat shock transcription factor. The orthologous gene in *Arabidopsis* (AT5G03720) encodes a *HEAT SHOCK FACTOR 3* (*HSFA3*), which is known to be involved in heat stress response (Li *et al.*, 2017). *HsfA3* is a vital heat-responsive transcription factor, which is controlled by *DREB2A*. It is also involved in conferring thermotolerance to plants during heat stress (Schramm *et al.*, 2008; Yoshida *et al.*, 2008). Interestingly, tomato HsfA3 (*SlHsfA3*), when overexpressed in *Arabidopsis* plants, provides thermotolerance upon heat stress (Li *et al.*, 2013). The up-regulation of *FvHSFA3* in both stages *Fragaria vesca* anthers suggests their role in heat stress response. Similar to the current findings, increased expression of heat shock transcription factors *HsfA2* and *HsfA3* were also observed in tomato male reproductive tissue (Frank *et al.*,

2009; Giorno *et al.*, 2013). Recently, identification and expression of heat shock factors was studied in *F. vesca* accession Heilongjiang-3. A total of 17 *FvHsf* were identified (Hu et al., 2015a). Vegetative tissue, especially old leaves, showed increased mRNA levels of *FvHsfA3a* along with *FvHsfA1b*, *FvHsfA4a*, *FvHsfA6a*, and *FvHsfC1* (Hu *et al*., 2015a). This study further indicates that expression of *FvHsfA3a* plays an important role in heat stress response restricted to vegetative tissues as compared to reproductive tissues. In contrast, another heat shock factor *HsfA2* is activated even at mild heat stress conditions (36 °C) for the protection of pollen grains in tomato (Giorno *et al.*, 2013). In the present study, higher expression of *FvHSFA3* gene in anther stages-6 and 8 indicates its involvement as a conventional transcription factor in response to heat stress. The data presented in this current study suggests similar heat responsiveness of *Fragaria vesca* anthers involving HSPs and HSFs as observed in other crop species.

3.5.3 Histone modification genes may contribute to thermotolerance during heat stress in anthers

Misregulation of histone constitutive and histone modification genes in *Fragaria vesca* anther stage-6 and stage-8 likely play an important role during heat stress. The expression of some of the conventional histone genes such as *HISTONE 2A* and *2B* was decreased in both meiotic and post-meiotic anther stages (Table 3.3 and Table 3.4). Similar results were supported in cotton anthers where about 24 histone constitution genes including *HISTONE H1*, *HISTONE H2A*, *HISTONE H2B*, *HISTONE H3*, and *HISTONE H4* were down-regulated in anthers (Min *et al.*, 2014).

Modulation of gene expression during developmental phases and environmental stress is also regulated via epigenetics (Hu *et al.*, 2015b; Jaenisch and Bird, 2003; Luo *et al.*, 2017).

Modification on histone involves addition or removal of methyl, acetyl, ubiquitination or phosphate on the histones (Strahl and Allis, 2000). The findings of this study demonstrate altered expression of genes involved in histone modifications such as deacetylation during heat stress. One of the genes that showed significantly higher expression in both stages was *HISTONE DEACETYLASE.* The data reveals increased expression of *FvH4_1g25790* (*FvHDA6*), whose homologue is *HISTONE DEACETYLASE 6* (*HDA6*) in *Arabidopsis*. Histone Deacetylases (HDACs) have been reported to play an important role during stress in regulating gene expression (Luo *et al.*, 2017). Recent study has shown that histone deacetylases are involved in heat stress response restricted to vegetative tissues of the plant. In *Arabidopsis*, higher expression of *HD2C* in vegetative tissue during heat stress (38 °C) suggests their involvement in heat stress response. It is demonstrated that HD2C together with BRAHMA (BRM)-containing SWITCH/SUCROSE NONFERMENTING regulate heat stress response (Buszewicz *et al.*, 2016). It is known also that functional interaction of HD2C and HDA6 regulates gene expression of stress-inducible genes (Luo *et al.*, 2012a; Luo *et al.*, 2012b).

Interestingly, past studies have revealed the role of histone deacetylase *HDA6* in basal heat thermotolerance. Mutant plants of *HDA6* exhibited misregulation of genes belonging to stress response, signaling, and protein processing (Popova *et al.*, 2013). Transcriptional regulation and chromatin processes were highly affected in the *hda6* mutant, suggesting a delayed transcriptional response to heat stress. The proposed underlying mechanism for such basic heat tolerance involves transcriptional gene silencing (Popova *et al.*, 2013). Overall increased expression of *FvHDA6* in stage-6 and stage-8 anthers of *Fragaria vesca* suggest their role in heat stress response via epigenetic regulation.

The higher expression of *FvH4_3g26570* (*FvHDA8*) gene was restricted to meiotic stage-6 anther of *Fragaria vesca*. Its *Arabidopsis* homologue *AT1G08460.1* or *HISTONE DEACETYLASE 8* represents one of the unclassified members of the RPD3-like superfamily of HDACs (Hollender and Liu, 2008; Luo *et al.*, 2017). The role of *HDA8* in plants is still not well understood. Some evidence shows the expression of the *HDA8* gene in *Arabidopsis* is restricted to late seed stage and pollen (Schmid *et al.*, 2005). This demonstrates that histone deacetylase 8 might be involved in regulating heat stress response in anthers, especially during early stages of anther development.

Although the function of histone modification genes in plants has been expanded, their role during heat stress is still emerging. Similar to this study, transcriptomic analysis was performed on heat-treated cotton anthers, where histone modification enzymes such as *HISTONE DEACETYLASES* (*HDA1* and *HDA2C*) were differentially expressed in anthers of heat-sensitive cotton line H05, thus suggesting the role of epigenetic modifications during heat stress restricted to male reproductive tissues (Min *et al.*, 2014). Based on current results, it may be deduced that the expression of histone modification genes during heat stress might be involved in the maintenance of DNA stability.

These results indicate the involvement of epigenetic histone modification in anthers during episodes of heat stress. The importance of epigenetic codes and their molecular mechanisms can help in identifying the pathways active during stress periods (Ueda and Seki, 2020). The kind of environmental stimulus that causes changes at such levels needs more investigation. However, the underlying mechanism by which histone modification regulates these processes is unknown in *Fragaria vesca*. There is very little information available about these modification enzymes during heat stress. Further examination of these epigenetic

modification genes can provide deeper insights into maintenance of DNA stability during heat stress affecting reproductive tissues.

3.5.4 Cell wall modification genes lead to anomalies in anther during heat stress

Altered expression of cell wall genes in stage-6 and stage-8 anther of *Fragaria vesca* is likely to be associated with heat stress response. Most of the genes involved in cell wall category were enriched in pectinesterase catalytic domain and glycoside hydrolase family (Table 3.5 and Table 3.6). The result obtained corroborates with the morphological phenotype obtained in Chapter 2. The effect of heat stress on cell wall modeling enzymes and proteins has not been well explored, but some studies report their involvement during heat stress. A similar observation regarding the reprogramming of cell wall proteins or gene clusters involved in cell wall biogenesis has been reported in a transcriptomics study of heat-stressed Chinese cabbage (*Brassica rapa* L.). Some of the cell wall encoding genes or proteins such as arabinogalactan protein, β-glucosidase, cellulose synthase, expansin, extensin, glycosyl transferase, pectinesterase, and xylosidase were found to be highly upregulated during high temperature conditions (37°C). The study further supported the importance of cell wall-remodeling enzymes in providing thermotolerance (Yang *et al.*, 2006).

The higher expression of glycoside hydrolase 16 family genes such as *FvH4_4g09230*, *FvH4_4g09250*, *FvH4_6g38190*, *FvH4_6g38150* and *FvH4_4g09160* during heat stress likely indicates their involvement in reprogramming of cell wall modeling. Their *Arabidopsis* homologue *AT4G25810.1* encodes for xyloglucan endotransglycosylase 6. Xyloglucan endotransglycosylase are a set of important cell wall modification enzymes that add xyloglucan chains via nonhydrolytic cleavage and then ligation to cell wall components (Eklöf and Brumer,

2010; Maris *et al.*, 2010). They play an important role in plant growth and development for instance cell expansion (Fry *et al.*, 1992; Hetherington and Fry, 1993). The wall of pollen mother cells (PMCs) is composed of cellulose, hemicellulose, and pectins (Brett and Waldron, 1996; Reiter, 1994). The disruption in any of the above-mentioned cell wall components and the enzymes associated with their stability will likely cause visible changes in the cell. The transcriptomics data of heat-treated meiotic and post-meiotic anthers is likely associated with misregulation of cell wall modifying enzymes such as xyloglucan endotransglycosylase. The involvement of xyloglucan endotransglycosylase has been studied before in heat-stressed durum wheat seedlings. Differential activity of xyloglucan endotransglycosylase was observed in apical roots of durum wheat, suggesting their role in heat stress (Iurlaro *et al.*, 2016). This further suggests that cell wall irregularities and disruption observed in tapetal cells and microspores (Chapter 2 of this dissertation) during heat stress indicates involvement of xyloglucan endotransglycosylase in heat stress response.

Another cell wall modeling enzyme that was significantly altered during heat stress is pectinesterase. Similarly, in tomato anthers, among the HsfA2-regulated genes, most of them involved genes related to cell wall remodeling enzymes such as pectin methylesterases, pectin acetylesterases, and pectate lyases (Fragkostefanakis *et al.*, 2016). Previously, the role of cell wall modifying enzymes has been shown in male reproductive development. *QUARTET* (*QRT*), a gene that encodes a pectin methylesterase (PME), is shown to be involved in pollen separation. Pollen grains of the mutant *qrt* fail to separate and are finally released as tetrads (Francis *et al.*, 2006). Another cell wall modifying enzyme, such as pectin acetylesterase, is also involved in maintaining physiochemical properties and cell wall extensibility. It was shown that overexpression of a pectin acetylesterase (PAE1) from black cottonwood (*Populus trichocarpa*)

in tobacco plants led to male sterility (Gou *et al.*, 2012). Environmental stress causes anomalies in cell walls (Degenhardt and Gimmler, 2000; Le Gall *et al.*, 2015; Yang *et al.*, 2006). The results obtained in this study indicate the importance of cell wall modification enzymes during heat stress restricted to male reproductive development. It is likely that the expression of cell wall modification genes could be involved in providing thermotolerance to plants.

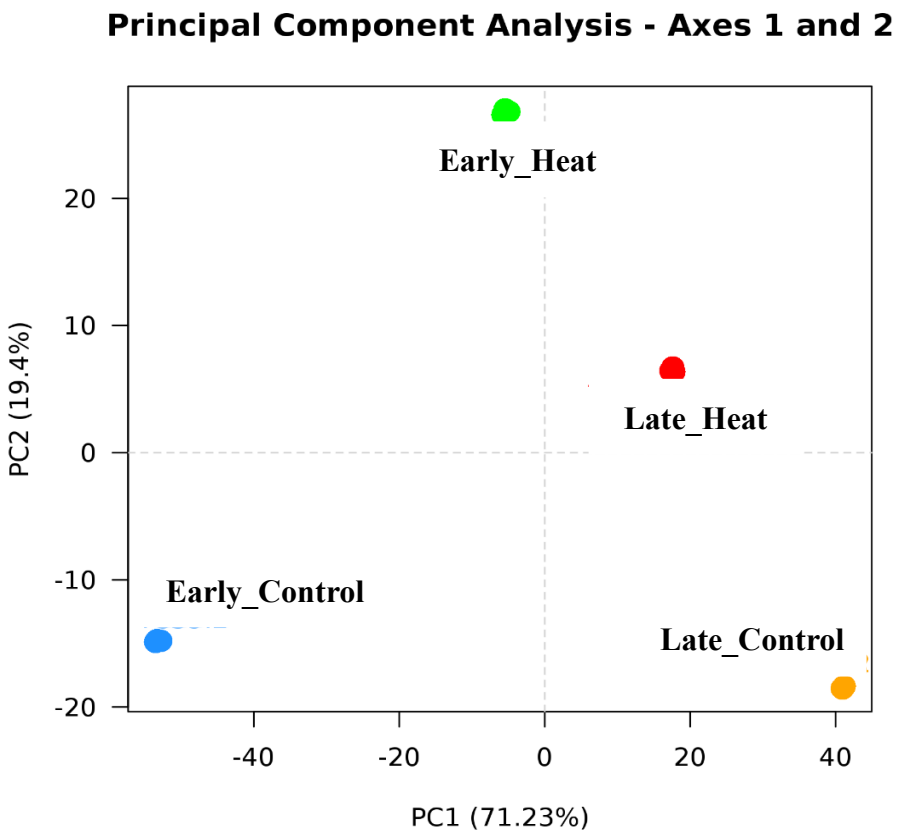


Figure 3.1 Principal component analysis on RNA-seq libraries of meiotic and post-meiotic anther stages under control and heat stress conditions. Each colored dot represented on the plot corresponds to one RNA-seq library. Triplicate samples were analyzed for both heat and control conditions. TCS5.1, TCS5.2, TCS5.3 represent RNA-seq libraries of control anther stage-6 (early) and TCS10.1, TCS10.2, TCS10.3 represent RNA-seq libraries of control anther stage-8 (late). Whereas THS5.1, THS5.2, THS5.3 represent RNA-seq libraries of heat-treated anther stage-6 (early). THS10.1, THS10.2, THS10.3 represents RNA-seq libraries of heat-treated anther at stage-8 (late). Blue and yellow color represents control early and late-stage anther libraries, respectively. Green and red color represents heat treated early and late anther libraries, respectively.

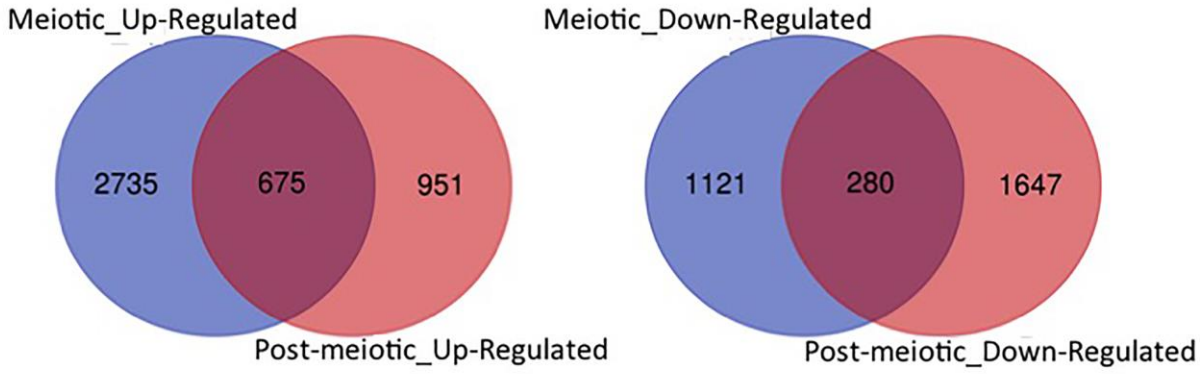


Figure 3.2 Differentially expressed genes in meiotic and post-meiotic anthers upon heat stress. Venn diagrams representing the numbers of heat responsive genes in meiotic (blue) and post-meiotic (pink) anther stages. *Fragaria vesca* buds at meiotic and post-meiotic anther stage were treated at 42°C for 48h for RNA-Seq analysis. Expression was compared to control (non-heat stressed) meiotic and control post-meiotic anthers. DEGs cut-off: log2 fold change ≥2 or ≤−2.

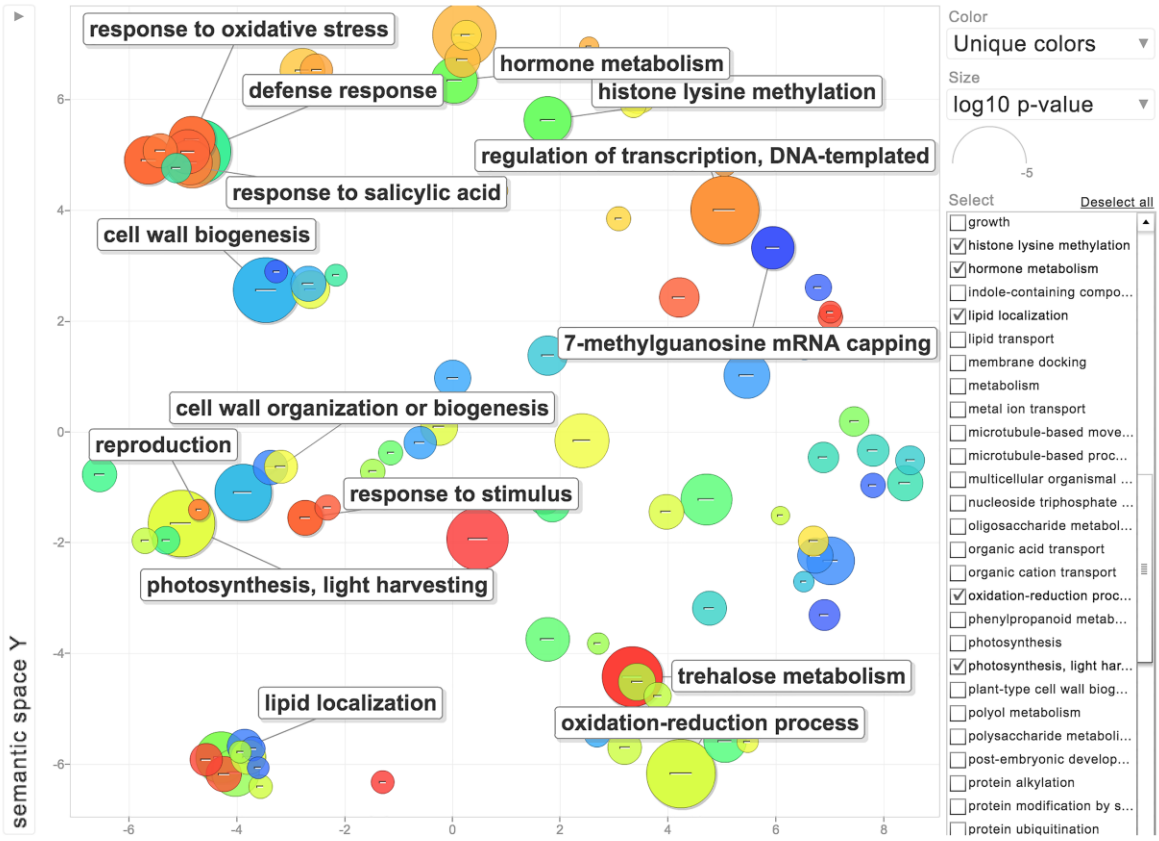


Figure 3.3 GO enrichment of meiotic anthers upon heat stress.

Scatter plot representing GO terms belonging to biological processes prevalent in meiotic anther stage during heat stress. Each bubble color corresponds to unique category. The size of the bubble represents enrichment of GO term based on the p-value.

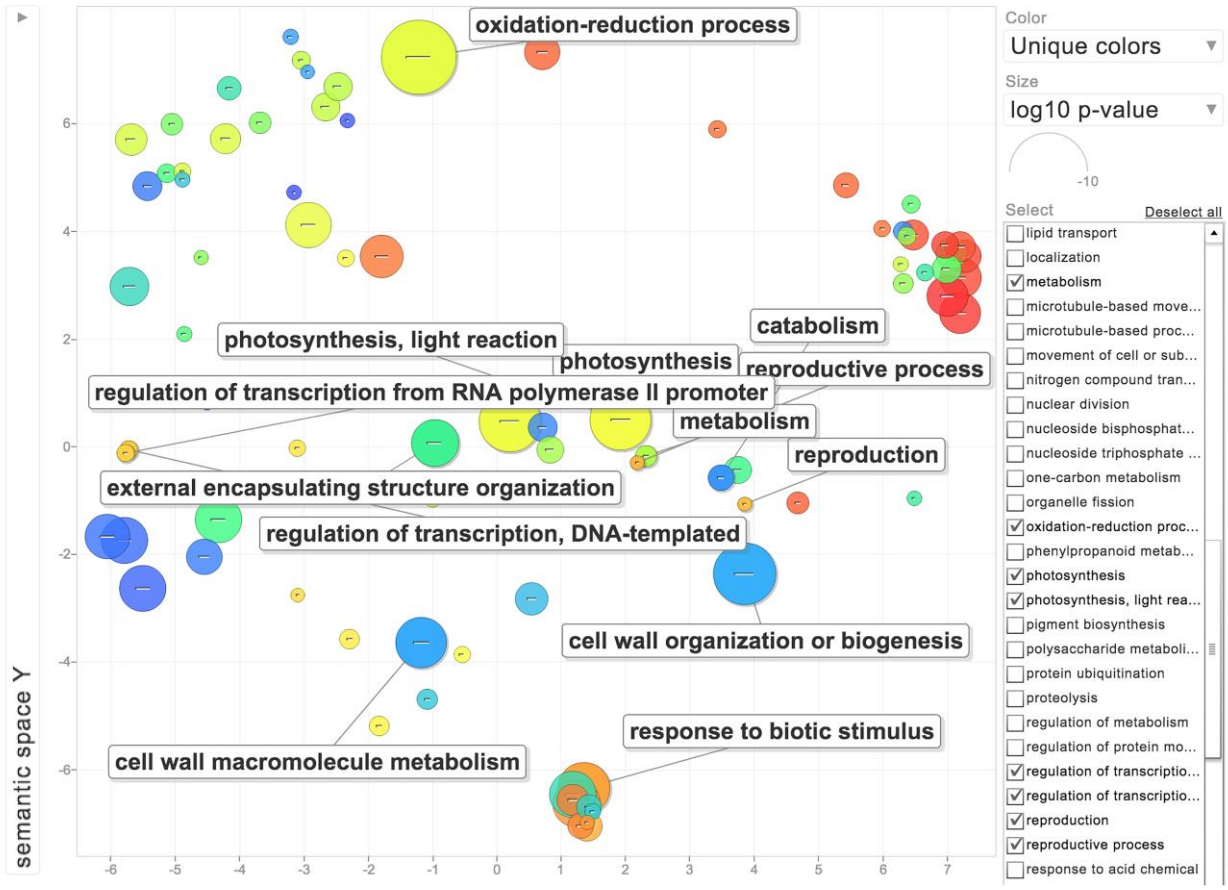
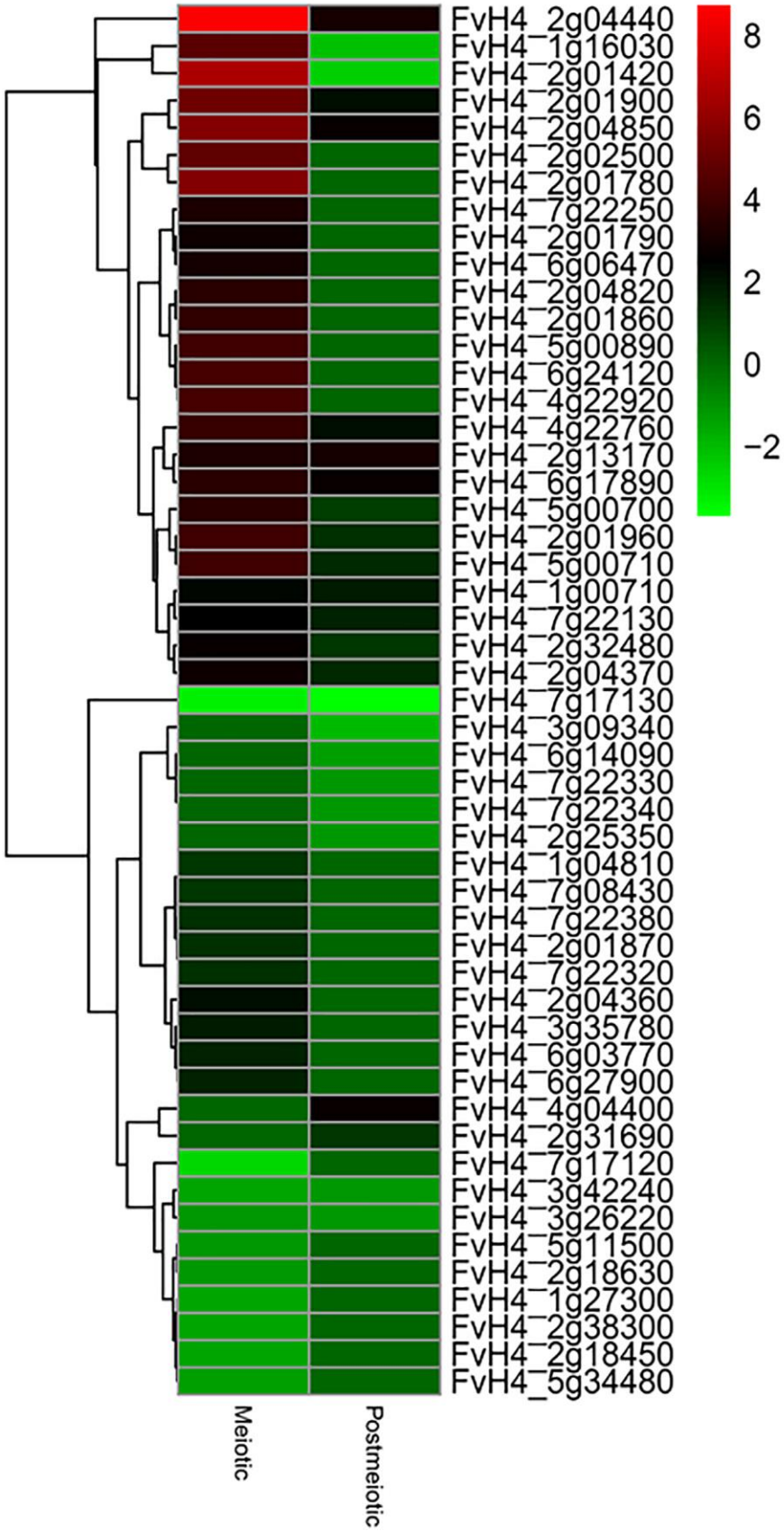


Figure 3.4 GO enrichment of post-meiotic anthers upon heat stress.

Scatter plot representing GO terms belonging to biological processes prevalent in post-meiotic anther stage during heat stress. Each bubble color corresponds to unique category. The size of the bubble represents enrichment of GO term based on the p-value.


FvH4_2g04440
FvH4_1g16030
FvH4_2g01420
FvH4_2g01900
FvH4_2g04850
FvH4_2g02500
FvH4_2g01780
FvH4_7g22250
FvH4_2g01790
FvH4_6g06470
FvH4_2g04820
FvH4_2g01860
FvH4_5g00890
FvH4_6g24120
FvH4_4g22920
FvH4_4g22760
FvH4_2g13170
FvH4_6g17890
FvH4_5g00700
FvH4_2g01960
FvH4_5g00710
FvH4_1g00710
FvH4_7g22130
FvH4_2g32480
FvH4_2g04370
FvH4_7g17130
FvH4_3g09340
FvH4_6g14090
FvH4_7g22330
FvH4_7g22340
FvH4_2g25350
FvH4_1g04810
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FvH4_7g22380
FvH4_2g01870
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FvH4_6g03770
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FvH4_3g42240
FvH4_3g26220
FvH4_5g11500
FvH4_2g18630
FvH4_1g27300
FvH4_2g38300
FvH4_2g18450
FvH4_5g34480
8
6
4
2
0
−2
Meiotic
Postmeiotic

Figure 3.5 Heat map representing heat responsive genes in meiotic and post-meiotic anthers.

Differential expression of heat-induced genes such as heat shock factors and heat shock proteins from meiotic and post-meiotic anther stages represented as log2fold change. Green color indicates down-regulation whereas red color indicates up-regulation. The clustering indicates closeness in genes based on log2fold change values.

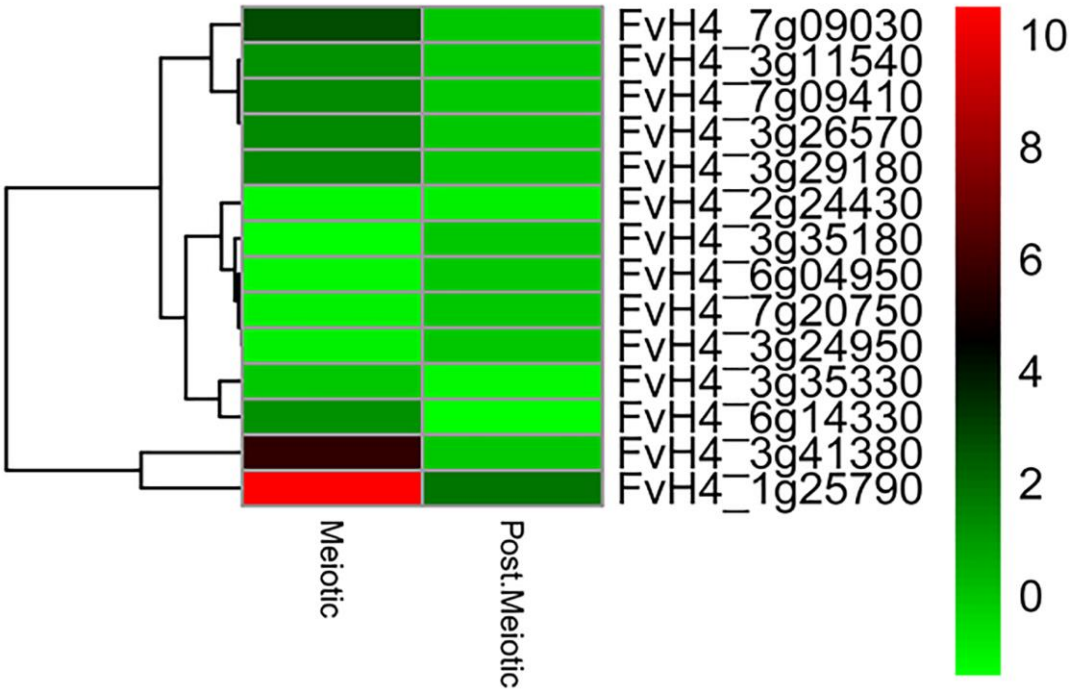

Figure 3.6 Heat map representation of altered expression of histone genes during heat stress in meiotic and post-meiotic stages of anther development.

Expression of histone genes from heat-treated meiotic and post-meiotic anthers represented as log2fold change. Green color indicates down-regulation whereas red color indicates up-regulation. The clustering indicates closeness in genes based on log2fold change values.

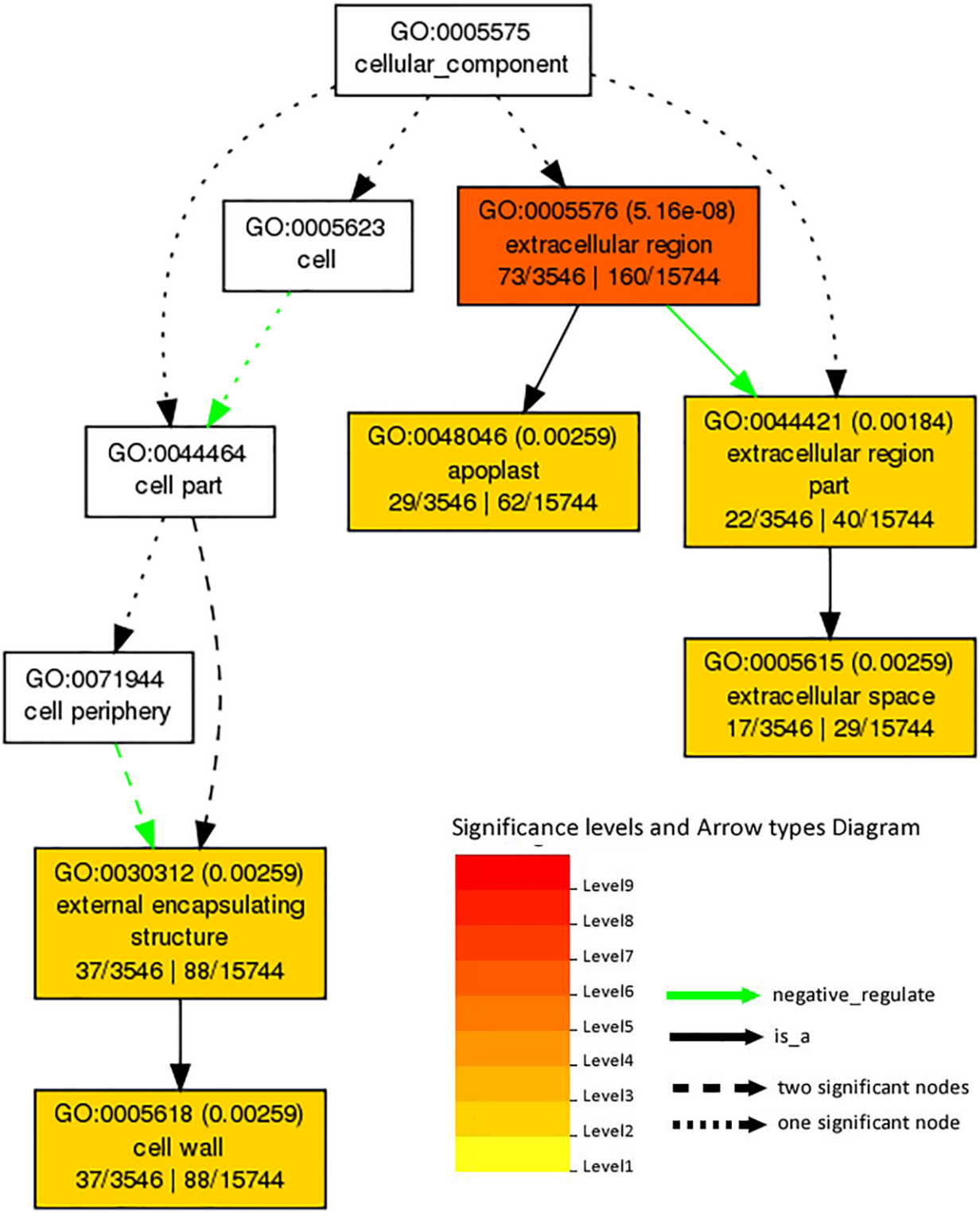
GO:0005575
cellular_component
GO:0005623
cell
GO:0005576 (5.16e-08)
extracellular region
73/3546 | 160/15744
GO:0044464
cell part
GO:0048046 (0.00259)
apoplast
29/3546 | 62/15744
GO:0044421 (0.00184)
extracellular region
part
22/3546 | 40/15744
GO:0071944
cell periphery
GO:0005615 (0.00259)
extracellular space
17/3546 | 29/15744
GO:0030312 (0.00259)
external encapsulating
structure
37/3546 | 88/15744
GO:0005618 (0.00259)
cell wall
37/3546 | 88/15744
Significance levels and Arrow types Diagram
Level9
Level8
Level7
Level6
Level5
Level4
Level3
Level2
Level1
negative_regulate
is_a
two significant nodes
one significant node

Figure 3.7 GO (Gene Ontology) analysis of heat stress induced cell wall genes in meiotic anther stage. The enrichment analysis of heat-treated meiotic anther stage-6 was performed using agriGO v2.0 (Tian *et al.*, 2017)**.** Each box represents a GO term along with its p-value in parenthesis associated with a specific category. The first pair of numeric values on the left side of the box represent heat-stressed genes detected during enrichment associated with a specific GO term along with the total number of genes fed in the input list. The second numeric value towards the right side of the box denotes the actual number of *Fragaria vesca* genes related to specific GO terms along with the total number of genes in *the Fragaria vesca* genome. Statistical significance is represented via box color.

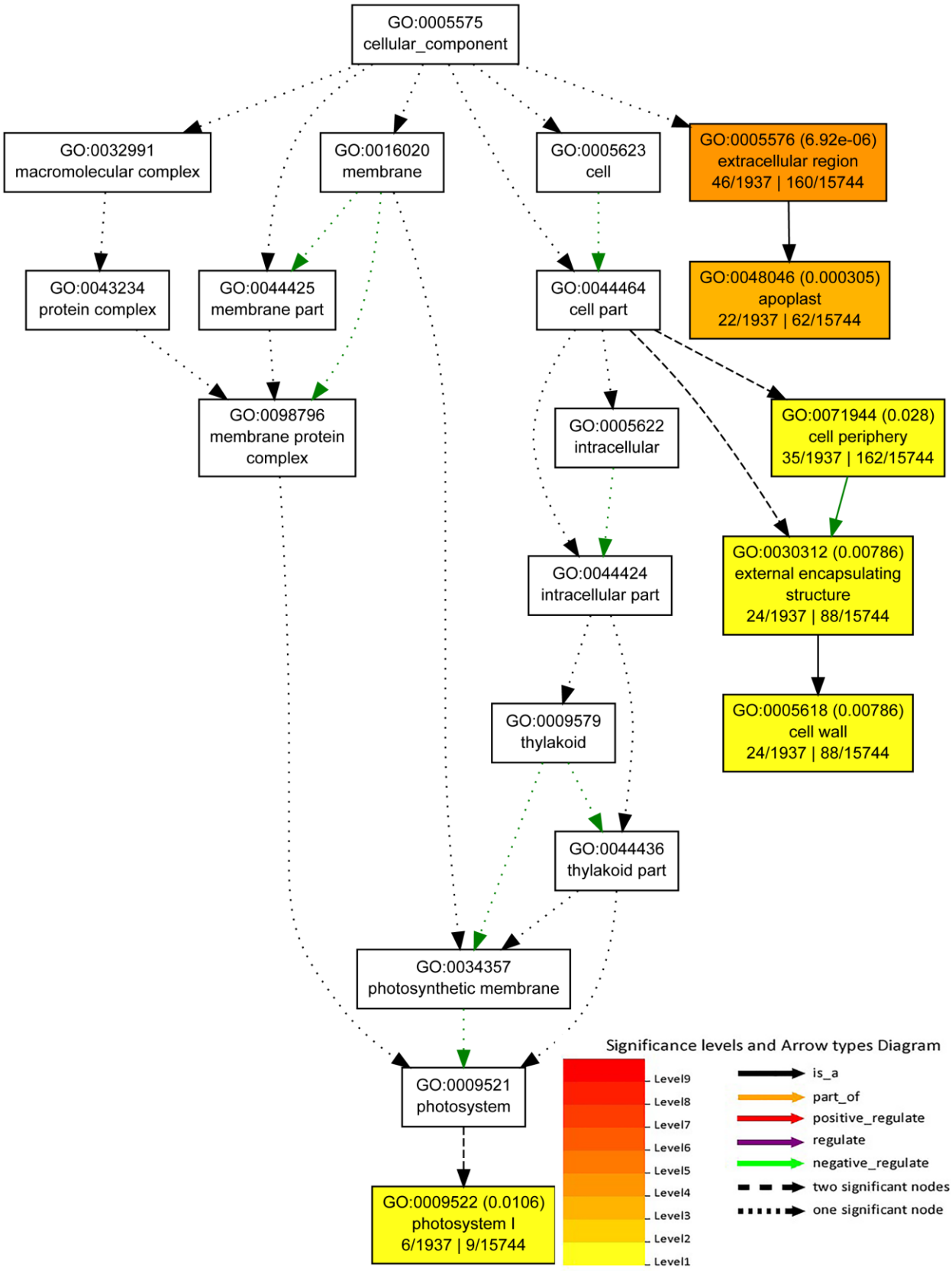
GO:0005575
cellular_component
GO:0032991
macromolecular complex
GO:0016020
membrane
GO:0005623
cell
GO:0005576 (6.92e-06)
extracellular region
46/1937 | 160/15744
GO:0043234
protein complex
GO:0044425
membrane part
GO:0044464
cell part
GO:0048046 (0.000305)
apoplast
22/1937 | 62/15744
GO:0098796
membrane protein
complex
GO:0005622
intracellular
GO:0071944 (0.028)
cell periphery
35/1937 | 162/15744
GO:0044424
intracellular part
GO:0030312 (0.00786)
external encapsulating
structure
24/1937 | 88/15744
GO:0009579
thylakoid
GO:0005618 (0.00786)
cell wall
24/1937 | 88/15744
GO:0044436
thylakoid part
GO:0034357
photosynthetic membrane
GO:0009521
photosystem
GO:0009522 (0.0106)
photosystem I
6/1937 | 9/15744
Significance levels and Arrow types Diagram
Level9
Level8
Level7
Level6
Level5
Level4
Level3
Level2
Level1
is_a
part_of
positive_regulate
regulate
negative_regulate
two significant nodes
one significant node

Figure 3.8 GO (Gene Ontology) analysis of heat stress induced cell wall genes in post-meiotic anther stage. The enrichment analysis of heat-treated post-meiotic anther stage-8 was performed using agriGO v2.0 (Tian *et al.*, 2017). Each box represents a GO term along with its p-value in parenthesis associated with a specific category. The first pair of numeric values on the left side of the box represent heat-stressed genes detected during enrichment associated with a specific GO term along with the total number of genes fed in the input list. The second numeric value towards the right side of the box denotes the actual number of *Fragaria vesca* genes related to specific GO terms along with the total number of genes in *the Fragaria vesca* genome. Statistical significance of enrichment is represented via box color.

Table 3.1: Differentially expressed heat shock genes between control and heat stressed meiotic anthers of *Fragaria vesca*

Gene Name	Gene Name version 2	Description	Log2FC	pvalue
FvH4_2g04440	gene34487	Heat shock protein 70 family	8.731	6.50E-13
FvH4_2g01420	gene07636	Heat shock protein 70 family	6.652	5.51E-07
FvH4_2g01780	gene07594	Heat shock protein 70 family	5.671	1.37E-04
FvH4_2g04850	#N/A	Heat shock protein 70 family	5.633	3.12E-18
FvH4_2g01900	gene34646	Heat shock protein 70 family	5.178	2.78E-08
FvH4_2g02500	#N/A	Heat shock protein 70 family	4.866	5.72E-04
FvH4_1g16030	gene24036	Heat shock factor (HSF)-type, DNA binding	4.617	8.59E-40
FvH4_4g22920	gene41116	Heat shock protein 70 family	4.233	2.12E-02
FvH4_6g24120	gene37681	Heat shock factor (HSF)-type, DNA-binding	4.202	1.24E-18
FvH4_5g00890	gene32313	Heat shock protein 70 family	4.026	1.00E-04
FvH4_5g00710	gene32293	Heat shock protein 70 family	3.975	2.07E-23
FvH4_2g01960	#N/A	Heat shock protein 70 family	3.959	4.99E-04
FvH4_4g22760	gene07289	Heat shock protein DnaJ, cysteine-rich domain	3.929	6.74E-54
FvH4_2g01860	#N/A	Heat shock protein 70 family	3.761	2.08E-02
FvH4_2g04820	gene39339	Heat shock protein 70 family	3.568	9.02E-03
FvH4_5g00700	gene32292	Heat shock protein 70 family	3.490	1.17E-08
FvH4_6g17890	gene30146	Heat shock factor (HSF)-type, DNA-binding	3.434	2.71E-25
FvH4_2g13170	#N/A	Heat shock protein Hsp90 family	3.188	1.17E-10
FvH4_7g22250	gene43220	Heat shock protein 70 family	3.183	1.98E-01
FvH4_6g06470	gene22159	Heat shock protein 70 family	2.983	7.65E-46
FvH4_2g04370	gene25573	Heat shock protein 70 family	2.838	3.76E-12
FvH4_2g01790	gene39274	Heat shock protein 70 family	2.788	1.04E-02
FvH4_2g32480	gene33887	Heat shock protein 70 family	2.724	2.05E-04
FvH4_7g22130	gene26631	Heat shock protein 70 family	2.462	6.81E-08
FvH4_1g00710	gene10105	Heat shock protein 70 family	2.331	1.06E-33
FvH4_2g04360	gene39333	Heat shock protein 70 family	2.223	9.25E-03
FvH4_3g35780	#N/A	Heat shock protein 70 family	1.888	8.35E-07
FvH4_6g27900	gene15872	Heat shock factor (HSF)-type, DNA-binding	1.718	4.63E-11
FvH4_6g03770	gene37508	Heat shock protein 70 family	1.689	8.05E-20
FvH4_7g22380	gene26613	Heat shock protein 70 family	1.427	1.43E-05
FvH4_2g01870	gene34650	Heat shock protein 70 family	1.367	7.38E-01
FvH4_7g22320	gene43222	Heat shock protein 70 family	1.367	7.38E-01

FvH4_7g08430	gene38173	Heat shock protein DnaJ, cysteine-rich domain superfamily	1.195	4.57E-42
FvH4_1g04810	gene30881	Heat shock factor (HSF)-type, DNA-binding	1.193	1.10E-07
FvH4_2g18630	gene17582	Heat shock protein DnaJ, cysteine-rich domain	-1.086	7.72E-21
FvH4_3g26220	gene23137	Heat shock protein DnaJ, cysteine-rich domain	-1.110	1.93E-42
FvH4_5g11500	gene25105	Heat shock protein 70 family	-1.163	2.82E-70
FvH4_5g34480	gene37411	Heat shock protein 70 family	-1.303	6.17E-82
FvH4_2g18450	gene17378	Heat shock protein Hsp90 family	-1.377	7.00E-01
FvH4_2g38300	gene08540	Heat shock protein Hsp90 family	-1.426	0.000
FvH4_3g42240	gene01495	Heat shock protein Hsp90 family	-1.457	0.000
FvH4_1g27300	gene31655	Heat shock protein 70 family	-1.474	6.70E-01
FvH4_7g17120	gene43114	Heat shock protein 70 family	-2.673	5.67E-09
FvH4_7g17130	gene43115	Heat shock protein 70 family	-3.357	8.13E-11

Table 3.2: Differentially expressed heat shock genes between control and heat stressed post-meiotic anthers of *Fragaria vesca*

Gene Name	Gene Name version 2	Description	Log2FC	pvalue
FvH4_2g13170	#N/A	Heat shock protein Hsp90 family	2.997	2.3E-14
FvH4_2g04440	gene34487	Heat shock protein 70 family	2.988	3.6E-19
FvH4_2g04850	#N/A	Heat shock protein 70 family	2.763	4.5E-20
FvH4_6g17890	gene30146	Heat shock factor (HSF)-type, DNA-binding	2.756	3.2E-28
FvH4_4g04400	gene11556	Heat shock protein Hsp90 family	2.672	1.6E-02
FvH4_2g01900	gene34646	Heat shock protein 70 family	2.168	7.0E-08
FvH4_4g22760	gene07289	Heat shock protein DnaJ, cysteine-rich domain	2.122	2.6E-34
FvH4_1g00710	gene10105	Heat shock protein 70 family	1.885	9.6E-33
FvH4_7g22130	gene26631	Heat shock protein 70 family	1.631	2.7E-03
FvH4_2g04370	gene25573	Heat shock protein 70 family	1.538	5.6E-07
FvH4_5g00710	gene32293	Heat shock protein 70 family	1.524	3.0E-14
FvH4_2g01960	#N/A	Heat shock protein 70 family	1.358	1.7E-02
FvH4_2g32480	gene33887	Heat shock protein 70 family	1.248	1.4E-02
FvH4_2g31690	gene02705	Heat shock factor (HSF)-type, DNA-binding	1.146	4.7E-09
FvH4_5g00700	gene32292	Heat shock protein 70 family	1.047	2.3E-03
FvH4_3g26220	gene23137	Heat shock protein DnaJ, cysteine-rich domain	-1.093	4.4E-132
FvH4_3g42240	gene01495	Heat shock protein Hsp90 family	-1.152	0
FvH4_7g22340	#N/A	Heat shock protein 70 family	-1.190	2.7E-07
FvH4_2g25350	gene10568	Heat shock factor binding 1	-1.192	1.0E-41
FvH4_7g22330	gene43223	Heat shock protein 70 family	-1.195	7.5E-01
FvH4_6g14090	gene18240	Heat shock protein 70 family	-1.336	0
FvH4_3g09340	gene02408	Heat shock factor (HSF)-type, DNA-binding	-2.023	2.4E-03
FvH4_1g16030	gene24036	Heat shock factor (HSF)-type, DNA-binding	-2.123	1.2E-109
FvH4_2g01420	gene07636	Heat shock protein 70 family	-2.471	3.0E-14
FvH4_7g17130	gene43115	Heat shock protein 70 family	-3.688	1.6E-02

Table 3.3: List of differentially expressed histone genes after meiotic anthers were subjected to heat stress

Gene Name	Gene Name version 2	Arabidopsis ID	Description	Log2FC	pvalue
FvH4_1g25790	gene12349	AT5G63110.1	Histone deacetylase superfamily	10.497	1.17E-18
FvH4_3g41380	gene01190	AT5G10980.1	Histone H3/CENP-A	5.661	0.000
FvH4_7g09030	#N/A	AT1G08880.1	Histone H2A	2.747	0.323
FvH4_3g26570	gene40220	AT1G08460.1	Histone deacetylase superfamily	1.367	0.738
FvH4_3g29180	gene15257		Transcription factor CBF/NF-Y/archaeal histone domain	1.367	0.738
FvH4_7g09410	gene04845	N/A	Linker histone H1/H5, domain H15	1.367	0.738
FvH4_3g11540	gene39975		Transcription factor CBF/NF-Y/archaeal histone domain	1.168	0.692
FvH4_6g14330	gene18214	AT2G28720.1	Histone H2B	1.141	5.26E-10
FvH4_3g24950	gene21782		Transcription factor CBF/NF-Y/archaeal histone domain	-1.050	1.25E-06
FvH4_7g20750	gene23356	AT5G59970.1	Histone H4	-1.078	2.28E-10
FvH4_2g24430	gene27760	AT1G08880.1	Histone H2A	-1.136	4.35E-32
FvH4_6g04950	gene22354	AT1G08880.1	Histone H2A	-1.230	9.03E-47
FvH4_3g35180	gene13945	AT1G07790.1	Histone H2B	-1.424	4.51E-53

Table 3.4: List of differentially expressed histone genes after post-meiotic anthers were subjected to heat stress

Gene Name	Gene Name version 2	Arabidopsis ID	Description	Log2FC	pvalue
FvH4_1g25790	gene12349	AT5G63110.1	Histone deacetylase superfamily	1.827	3.30E-26
FvH4_2g24430	gene27760	AT1G08880.1	Histone H2A	-1.023	1.00E-85
FvH4_3g35330	gene14169	AT5G02560.1	Histone H2A	-1.164	6.80E-202
FvH4_6g14330	gene18214	AT2G28720.1	Histone H2B	-1.306	9.52E-62

Table 3.5: List of differentially expressed cell wall genes during heat stress in meiotic stage-6 anthers of *Fragaria vesca*

Gene Name	Gene Name Version 2	Description	Log2FC	pvalue
FvH4_6g38170	gene28698	Glycoside hydrolase family 16	4.311	0.000
FvH4_4g09150	gene05205	Glycoside hydrolase family 16	3.832	0.018
FvH4_5g18880	gene08938	Pectinesterase, catalytic	-1.109	1.58E-07
FvH4_6g16550	gene09672	Glycoside hydrolase family 16	-1.947	9.35E-24
FvH4_1g09610	gene12966	Pectinesterase, catalytic	1.365	5.62E-30
FvH4_1g18550	gene05591	Glycoside hydrolase family 16	1.455	9.46E-31
FvH4_3g02630	gene19553	Glycoside hydrolase family 16	-1.090	8.89E-05
FvH4_4g33970	gene06194	Pectinesterase, catalytic	1.357	0.002
FvH4_7g19920	gene23206	Pectinesterase, catalytic	2.115	0.554
FvH4_5g35590	gene12105	Pectinesterase, catalytic	-1.365	0.017
FvH4_4g15880	gene36685	Pectinesterase, catalytic	1.794	0.464
FvH4_3g00840	gene19781	Glycoside hydrolase family 16	2.018	1.63E-23
FvH4_4g12960	gene06605	Glycoside hydrolase family 16	2.254	3.30E-37
FvH4_6g17090	gene00093	Pectinesterase, catalytic	1.857	2.38E-21
FvH4_1g24090	gene17879	Pectinesterase, catalytic	-1.119	2.48E-33
FvH4_1g09290	gene12930	Pectinesterase, catalytic	5.850	6.21E-05
FvH4_4g06700	gene24855	GDSL lipase/esterase	-3.658	3.8E-176
FvH4_6g38150	gene37832	Glycoside hydrolase family 16	3.360	4.87E-12
FvH4_7g11320	gene03929	Pectinesterase, catalytic	5.218	0.001
FvH4_6g17430	gene30095	Pectinesterase, catalytic	4.728	0.002
FvH4_1g06350	gene11323	Pectinesterase, catalytic	-2.898	3.1E-100
FvH4_6g38190	gene28700	Glycoside hydrolase family 16	3.701	1.23E-05
FvH4_4g18680	#N/A	Pectinesterase, catalytic	5.489	0.000
FvH4_2g18760	gene17597	Glycoside hydrolase family 16	7.500	3.37E-81
FvH4_3g30850	gene24600	Glycoside hydrolase family 16	-2.054	4.41E-30
FvH4_4g09230	gene05204	Glycoside hydrolase family 16	6.412	6.35E-21
FvH4_4g09160	gene05220	Glycoside hydrolase family 16	6.247	1.99E-23
FvH4_4g09250	gene05197	Glycoside hydrolase family 16	2.280	0.467
FvH4_6g39480	gene24221	Pectinesterase, catalytic	1.367	0.738
FvH4_7g19540	gene23297	Pectinesterase, catalytic	1.367	0.738
FvH4_3g36820	gene30193	Pectinesterase, catalytic	4.647	0.007
FvH4_5g35580	gene12106	Pectinesterase, catalytic	1.890	0.410
FvH4_7g04220	gene21487	Pectinesterase, catalytic	4.470	0.011
FvH4_4g22090	gene00216	Glycoside hydrolase family 16	1.359	1.39E-63
FvH4_4g18670	gene07045	Pectinesterase, catalytic	4.920	0.002
FvH4_7g11340	gene03924	Pectinesterase, catalytic	3.016	0.278
FvH4_5g23180	gene13718	Glycoside hydrolase family 16	1.827	4.03E-12

Table 3.6: List of differentially expressed cell wall genes during heat stress in post-meiotic anthers of *Fragaria vesca*

Gene Name	Gene Name Version 2	Description	Log2FC	pvalue
FvH4_5g14710	gene26037	Pectinesterase, catalytic	-1.761	1.30E-11
FvH4_1g09560	gene12962	Pectinesterase, catalytic	-2.312	0.003
FvH4_3g30850	gene24600	Glycoside hydrolase family 16	-2.342	1.89E-15
FvH4_4g09230	gene05204	Glycoside hydrolase family 16	1.731	4.90E-09
FvH4_4g09160	gene05220	Glycoside hydrolase family 16	3.084	3.90E-20
FvH4_4g09250	gene05197	Glycoside hydrolase family 16	1.647	0.577
FvH4_3g29490	gene23525	Pectinesterase, catalytic	-3.129	6.49E-230
FvH4_2g25960	gene10629	Pectinesterase, catalytic	-3.390	0.010
FvH4_6g43860	gene01986	Glycoside hydrolase family 16	-1.105	4.81E-259
FvH4_4g18690	gene07048	Pectinesterase, catalytic	-1.549	1.89E-02
FvH4_4g18670	gene07045	Pectinesterase, catalytic	-2.540	4.49E-03
FvH4_5g35570	gene12107	Pectinesterase, catalytic	-1.195	7.62E-01
FvH4_4g12140	gene22434	Pectinesterase, catalytic	-2.211	6.76E-04
FvH4_6g38190	gene28700	Glycoside hydrolase family 16	2.761	1.93E-10
FvH4_6g17430	gene30095	Pectinesterase, catalytic	4.873	0.001
FvH4_4g34630	gene00661	Glycoside hydrolase family 16	1.447	0.003
FvH4_1g03250	gene31035	Pectinesterase, catalytic	1.689	0.591
FvH4_7g11320	gene03929	Pectinesterase, catalytic	-1.592	0.012
FvH4_1g06350	gene11323	Pectinesterase, catalytic	-2.113	4.50E-115
FvH4_4g18680	#N/A	Pectinesterase, catalytic	-1.880	2.19E-05
FvH4_2g18760	gene17597	Glycoside hydrolase family 16	-1.576	0
FvH4_2g25980	gene11075	Pectinesterase, catalytic	-1.498	2.31E-11
FvH4_6g38150	gene37832	Glycoside hydrolase family 16	2.210	2.26E-09
FvH4_4g06700	gene12291	Glycoside hydrolase family 16	1.681	2.39E-40

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Chapter 4

Functional analysis of *Arabidopsis HSFA3, NAC029* and *HDA6* genes in response to heat stress

4.1 Abstract

Exposure to high temperature causes severe damage to reproductive development in plants. In this study, three representative genes identified from the RNA-seq analysis were chosen to test their function in heat stress using the model plant *Arabidopsis*. The *Arabidopsis* T-DNA knockout mutants of *Fragaria vesca* orthologous genes were treated at 42°C for 6 hours and their silique length was analyzed to evaluate changes in fertility. After heat stress, *hsaf3* (*heat shock factor a3*), *nac029* (*nam, ataf, and cuc 29*), and *hda6* (*histone deacetylase 6*) mutant plants showed reduced silique length in comparison with the wild-type plant, although high temperature-induced sterility was significantly increased in the *hda6* mutant than that in *hsaf3* and *nac029* mutants. The results suggest that HSFA3 and NAC029 transcription factors as well as the HDA6 histone modification factor play important roles in heat stress response during plant reproduction via both genetic and epigenetic control.

4.2 Introduction

Crop production worldwide is highly affected by heat stress. The global mean temperatures projected by Intergovernmental Panel for Climate Change (IPCC) predicts an increase of about 3.7±1.1°C by the end of this century (Field *et al.*, 2014; Houghton *et al.*, 2001). Extreme high temperatures will reduce cereal production for about 6 to 7% yield per 1°C increase in weather (Lesk *et al.*, 2016). High temperature stress affects plants during vegetative as well reproductive

phases of development. Of the two phases of development, the reproductive phase is more affected during episodes of heat stress (Giorno *et al.*, 2010). Some of the crops affected in terms of yield as a consequence of heat stress include wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and rice (*Oryza sativa*) (Barnabás *et al.*, 2008). In order to mitigate the deleterious effects of heat stress on plants, a more comprehensive understanding of molecular mechanisms underlying thermotolerance is needed.

Plants use distinct mechanisms to combat high temperature stress. The heat shock response has been widely studied in plants at vegetative stages. Heat shock response activates a cascade of heat shock factors (HSFs) resulting in induction of heat shock proteins (HSPs) (Baniwal *et al.*, 2004; Kotak *et al.*, 2007). The complex mechanisms alleviating heat damage include maintenance of membrane stability, calcium dependent signaling, unfolded protein response (UPR), production of reactive oxygen species (ROS), and phytohormone signaling (Kotak *et al.*, 2007; Mittler *et al.*, 2012; Wahid *et al.*, 2007). HSPs play an important role as molecular chaperons in stabilizing and refolding the misfolded proteins (Vierling, 1991). One of the most vital components of heat shock response is the HSFA1 transcription factor, which forms a complex with HSFA2 to regulate expression of heat responsive genes (Mishra *et al.*, 2002; Scharf *et al.*, 2012).

The unfolded protein response (UPR) is initiated in response to aggregation of misfolded proteins. Many membrane-associated transcription factors such as basic leucine zipper (bZIP), NAC [NAM (NO APICAL MERSITEM), ATAF, and CUC (CUP-SHAPED COTYLEDON)] are involved in the UPR cascade (Liu and Howell, 2010; Yang *et al.*, 2014a; Yang *et al.*, 2014b). Emerging evidence suggests that bZIP28 and bZIP60 play an important role in providing thermotolerance at the reproductive stage (Zhang *et al.*, 2017).

Another essential mechanism regulating gene expression in many biological processes is epigenetic control (Holoch and Moazed, 2015; Kouzarides, 2007). Various epigenetic regulators participate in stress responses (Kumar and Wigge, 2010; Ueda and Seki, 2020). In *Arabidopsis*, prolonged exposure to elevated temperatures causes epigenetic silencing of transgenes and transposons (Lang-Mladek *et al.*, 2010). For instance, during heat stress response both HD2C deacetylase and BRAHMA (BRM), a SWI/SNF chromatin remodeling ATPase, regulate heat stress responsive genes in *Arabidopsis* (Buszewicz *et al.*, 2016). Moreover, *GCN*, a histone modification gene, regulates heat stress by conferring thermotolerance to *Arabidopsis*. It regulates the heat stress response via enrichment in the promoter regions of *HEAT SHOCK TRANSCRIPTION FACTOR A3* and *ULTRAVIOLET HYPERSENSITIVE6* genes (Hu *et al.*, 2015b). The *HDA6* gene, encoding a histone deacetylase, is also involved in transcriptional regulation of heat-specific genes (Popova *et al.*, 2013). These studies suggest the importance of transcription factors (such as HSF and bZIP) and epigenetic regulators (such as histone deacetylase) in heat stress at the vegetative phase of development, whereas their roles at the reproductive phase are not clear.

In Chapter 3, the transcriptomics study of heat-treated anthers of *Fragaria vesca* identified genes involved in heat stress responses (Chapter 3). Currently, there are no mutants available for *Fragaria vesca* to explore the role of essential genes involved in heat stress. An alternative approach was to use available mutants in *Arabidopsis* and target candidate analogous genes identified in *F. vesca.* These genes were the focus for testing their functionality in heat stress response using T-DNA insertion mutants in the model plant *Arabidopsis* (Krysan *et al.*, 1999). Candidate genes encoding transcription factors (e.g., HSFA3 and NAC029) and histone deacetylases (e.g., HDA6), were examined for their role in heat stress. Most of

the *Arabidopsis* mutants used in this study have not been explored to identify the role of mutated genes in heat stress related to reproductive development. This represents both a new examination for *Arabidopsis* and application to understanding heat stress also in *F. vesca*. Suggesting that both genetic and epigenetic regulation are important for plants to cope with heat stress during reproduction.

4.3 Materials and Methods

4.3.1 *Arabidopsis* lines and growing conditions

All *Arabidopsis thaliana* plants used in these experiments are in the Columbia ecotype background. The wild-type plant used is Col-0. All T-DNA insertion lines, including SALK_011107C (*hsfa3*), SALK_005010C (*nac029*) and SALK_201895C (*hda6*) were obtained from the Arabidopsis Biological Resource Center. For phenotypic analysis seeds were sown in moist SUN GRO Metro-Mix 360 (12 seeds per one square pot with 12cm L X 12cm B X 5cm H dimensions) and stratified at 4°C for 2 days. After stratification they were transferred to a growth chamber with 23°C temperature setting and 16/8-h day/night photoperiod.

4.3.2 Identification of *HSFA3, NAC029* and *HDA6* mutants

After the emergence of 5-6 leaves, leaf tissue was collected for genotyping of individual mutants (wild type as control). Genomic DNA was isolated using Edwards buffer. Every mutant was checked for their respective mutations by performing PCR using gene-specific and T-DNA specific primers. The T-DNA insertion site for each mutant is shown in Fig. 4.1. All the PCR primer sequences are listed in Table 4.1. PCR reactions were carried out using a Bio-Rad thermo cycler. GoTaq master mix (Promega Catalogue No. M7122) was used for PCR.

4.3.3 Heat stress treatment

Heat stress was performed on reproductive stage plants (bolting stage i.e. appearance of first flowers) as previously described (Zhang *et al.*, 2017). After the appearance of inflorescences, plants were exposed to 42°C high temperature for 6 hours in a growth chamber under a 16-h light/8-h dark photoperiod with light intensity of 190 lux and 50% humidity. For comparison, control plants were grown at 23°C at the same stage. Before heat treatment the oldest bud in an inflorescence was marked with thin tape in order to track their development after exposure to heat stress. Following heat stress, plants were transferred back to a growth chamber set at control temperature of 23°C and were left to grow for 10-14 days.

4.3.4 Data collection and analysis

Following the recovery period, about 8-10 individual plants were taken for silique analysis. The lengths of siliques derived from the marked bud and the next thirteen siliques from younger buds in each inflorescence were measured using a ruler. Siliques were categorized into three types based on the length (Zhang *et al.*, 2017). Silique length >10mm was considered as being fully fertile, followed by 5-10mm as partially sterile and, <5mm as completely sterile (Zhang *et al.*, 2017). For statistical analysis two-way ANOVA was used to distinguish the phenotypic differences. Statistics were calculated using MS Excel software. Results for silique length and fertility are presented as mean ± Standard Deviation. Two-way ANOVA was used to distinguish the phenotypic differences for silique length and position. For silique fertility, One-way ANOVA was used to compare fertile, partially sterile, and sterile siliques for WT and mutant. Values were considered statistically significant at $P < 0.05$.

4.3.5 Gene ID conversion and functional enrichment analysis

To use *Arabidopsis* to examine functions of *Fragaria vesca* genes identified by RNA-seq analysis, the *Fragaria vesca* gene IDs were converted into *Arabidopsis* gene IDs and then the functional enrichment analysis was conducted. *Fragaria vesca* gene IDs were converted to *Arabidopsis* gene IDs using blastx_Fragaria_vesca_v4.0.a1_vs_TAIR10.xlsx file from the Genome Database for Rosaceae (GDR) website: ftp://ftp.bioinfo.wsu.edu/species/Fragaria_vesca/Fvesca-genome.v4.0.a1/homology/. Gene IDs were manually matched in an Excel worksheet. For downstream analyses of genes, enrichment analysis was performed using DAVID - Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov/) (Sherman and Lempicki, 2009). Enrichment maps representing key gene clusters were built using the Cytoscape network visualization software (Merico *et al.*, 2010).

4.4 Results

4.4.1 Functional enrichment analysis of gene clusters involved in heat stress response

The Cytoscape network visualization software generated about 110 enriched gene sets (linked by blue lines) from *Arabidopsis* IDs converted from the *Fragaria vesca* gene IDs. The enriched gene-clusters were manually circled to depict associated biological and molecular processes. One of the prevalent enriched gene set clusters was comprised of GO:0009768, GO:0009765 terms belonging to photosynthesis and light harvesting in photosystem I, respectively (Fig. 4.1). GO: 0055114 term associated with oxidation-reduction processes was also a predominant node. Gene clusters such as transcription regulation, mRNA processing, cell wall, and response to heat stress were also observed as separate clusters. All these clusters represent strong correlation of gene

sets to heat stress response. The current data overlaps with the RNA-Seq data observed in Chapter 3.

Based on functional enrichment analysis and the log fold change of gene expression value, three representative genes were chosen to test their functions in heat stress response using *Arabidopsis* (Table 4.2).

4.4.2 Identification of *hsfa3, nac029,* and *hda6* mutants

The T-DNA insertion lines obtained for *HSFA3* (AT5G03720), *NAC029* (AT1G68490), and *HDA6* (AT5G63110) genes in Columbia-0 background (Fig. 4.2). *hsfa3*, *nac029,* and *hda6* lines were confirmed as homozygous mutants by PCR (Fig. 4.2). Seeds were amplified from homozygous plants for further study.

4.4.3 *HSFA3* loss-of-function mutant showed hypersensitivity to high temperature stress at the reproductive stage

In the *hsfa3* mutant, it was observed that silique length from the 1st to 8th positions was highly reduced as compared to wild type upon heat stress (Fig. 4.3 A to D; Table 4.3) (Two-way ANOVA, $p<0.0006$). As the siliques were divided into three categories (Fig. 4.3E), there was an overall reduction observed in fertility of heat stressed plants (Fig. 4.3F; Table 4.4). The percentage of fertile siliques was reduced in *hsfa3* mutant (71.4%) as compared to wild-type plants (83.9%) (One-way ANOVA, $p<0.01$). In contrast to fertile siliques of the *hsfa3* mutant, a higher percentage of partially sterile and sterile siliques was observed (Fig 4.3F) (One-way ANOVA, $p<0.004$ for partially sterile siliques and $p<0.2$ for sterile siliques). These results

suggest that *hsfa3* mutant plants are more sensitive to heat stress than wild-type plants at the reproductive stage.

4.4.4 *NAC029* loss-of-function mutant showed hypersensitivity to high temperature stress at the reproductive stage

Similar to the *hsfa3* mutant, reduction of silique lengths was also observed in *nac029* mutant plants subjected to heat stress. The silique length was decreased from the 1st silique through 8th silique in the *nac029* mutant as compared to the wild-type (Fig. 4.4 A to C; Table 4.5) (Two-way ANOVA, $p<0.02$). In silique positions from 9 through 14, an almost similar length trend was obtained for wild-type and mutant *nac029* (Fig. 4.4C; Table 4.5). Moreover, the overall inflorescence fertility of the *nac029* mutant was reduced relative to the wild-type plants (Fig. 4.4D; Table 4.6). The percentages of partially sterile (14.28%) and fully sterile siliques (17.85%) were higher in *nac029* mutant in contrast to the wild-type where 8.9% were partially sterile, and 7.14% were sterile (Fig. 4.5D) (One-way ANOVA, $p<0.2$ for partially sterile siliques and $p<0.005$ for sterile siliques). These results suggest the involvement of the novel transcription factor NAC029 in regulating thermotolerance during reproductive development.

4.4.5 *HDA6* knockout mutation leads to sterility due to heat sensitivity

The *hda6* mutant was highly sensitive to heat stress (Fig. 4.5 A to C; Table 4.7). There was a significant reduction in the silique length starting from the 1st position through the 14th position, compared to the wild type plants (Fig. 4.5C) (Two-way ANOVA, $p<0.0001$). The percentage of fertile siliques in the *hda6* mutant was much reduced (8.03%) compared to wild-type (83.92 %) (Fig. 4.5D; Table 4.8) (One-way ANOVA, $p<0.0001$). Moreover, the *hda6* mutant exhibited a

higher percentage of partially sterile (36.6%) and sterile siliques (62.5%) as compared to wild-type plants (Fig. 4.5D) (One-way ANOVA, $p<0.0001$ for both partially sterile and sterile siliques). These results suggest that the histone modification gene *HDA6* is important in heat stress response associated with reproduction.

4.5 Discussion

4.5.1 Heat shock transcription factor (HSFA3) is involved in heat stress during reproduction

The role of selected genes in heat stress response was studied here by analyzing heat stress-induced phenotypes of corresponding *Arabidopsis* mutants. Sterility is observed in *Arabidopsis* plants at high temperatures (Bac-Molenaar *et al.*, 2015; Sakata *et al.*, 2010). The results showed that the *hsfa3* mutant was more sensitive to heat stress in reproduction. Reduction in silique length is likely associated with improper fertilization leading to failure in seed production. In addition, this study demonstrated that flowers at late stages (approximately flowering stage 12 to stage 9) (Sanders *et al.*, 1999; Smyth *et al.*, 1990) are also vulnerable to high-temperature stress. Heat stress (42°C/4 h) in *Arabidopsis* caused short siliques at flower positions 1 to 3 and 10 to 13 as a consequence of failure in seed production (Kim *et al.*, 2001). A similar study on *Arabidopsis* demonstrated that heat exposure at 35°C for 1 day causes reduction in silique length and sensitivity of floral stage 9, 10, 11 and 12 (Bac-Molenaar *et al.*, 2015). Hence heat stress impairs fertility by affecting flower development specific to stage 9 through 12 when male meiosis, microspore and pollen development are active.

HSFs have been previously shown to be involved in heat stress response. Multiple genes represent the HSF family, which have similar functions (Guo *et al.*, 2016). Thus, studying the

role of single knockout mutants provides in-depth insight into heat stress pathways. *HSFA1* and *HSFA2* are key regulators involved in heat shock response (Charng *et al.*, 2007; Liu *et al.*, 2011). *HSFA2* works downstream of *HSFA1* in the same regulatory pathway (Liu and Charng, 2013). The *hsfa2 hsfa3* double mutant was highly heat sensitive and showed reduced survival rates, suggesting that both *HSFA2* and *HSFA3* are important for thermotolerance (Li *et al.*, 2017). However, the role of *HSF* genes in heat stress response at the reproductive stage remains unknown. This functional study revealed the important role of *HSFA3* in response to high temperature during reproduction. It will be worthwhile to test whether defects in male and female reproductive organs caused sterility under heat stress in this line.

4.5.2 *NAC029*- a novel regulator in heat stress response at the reproductive stage

Although a few studies have been performed on transcription factors associated with heat stress, the present data demonstrate *NAC029* as a novel candidate regulating the heat stress response. NAC transcription factors, consisting of NAC (NAM /ATAF1, 2/CUC2), are known to play important roles in plant development as well as abiotic stress (Aida *et al.*, 1997; Aida *et al.*, 1999; Souer *et al.*, 1996). *ANAC019* and *ANAC092* are associated with drought tolerance and salt stress response, respectively (He *et al.*, 2005; Tran *et al.*, 2004). *JUNGBRUNNEN1* (*JUB1*; *ANAC042*) is crucial for heat tolerance and thermopriming. The *jub1-1* mutant displayed heat sensitivity as compared to its over-expression lines (Shahnejat-Bushehri *et al.*, 2012; Wu *et al.*, 2012). Similarly, NAC transcription factors from wheat (*TaNAC2L*) and rice (*SNAC3*; *ONAC003*, LOC_Os01g09550) are involved in heat tolerance at the seedling stage (Fang *et al.*, 2015; Guo *et al.*, 2015). Upon heat stress, flowers of the *nac019* mutants exhibited sensitivity to heat stress (Guan *et al.*, 2014). Additionally, expression of *NAC019* was higher in flowering

stage in wild-type plants. Moreover, *NAC019* overexpression lines depicted an increased level of heat responsive genes, suggesting its positive role in heat stress in reproductive tissues. An elevated level of the *FvNAC029* gene expression was observed in *Fragaria vesca* anthers under heat stress. The data of this study demonstrates that *Arabidopsis nac029* single mutant displayed a significantly increased sensitivity to heat stress at the reproductive stage when compared to wild-type. Thus, *NAC029* might serve as a novel regulator to maintain fertility under heat stress.

4.5.3 Histone modification may regulate fertility during heat stress

The results showed that *HDA6* plays a vital role in thermotolerance at the reproductive stage. The loss-of-function *hda6* mutant exhibited significant reduction in silique length. The results of this study show that at the extreme heat stress of 42°C *hda6* mutants were almost completely sterile compared to wild-type plants which still produced fertile siliques. It further demonstrated that most of the flowers of *hda6* mutant at all developmental stages were hypersensitive to heat stress. Although previously *hda6* plants have been shown to be sensitive to heat stress at the vegetative stage (Popova *et al.*, 2013), these findings provide new evidence about its role in heat response at the reproductive stage.

Histone deacetylases form part of the multiprotein nuclear complexes which aid in gene regulation via transcriptional repression (Yang and Seto, 2008). Very few studies have been performed on the role of histone deacetylase in heat stress. In *Arabidopsis*, HD2C deacetylase interacts with the chromatin-remodeling factor BRAHMA (BRM). Upon heat stress *HD2C* transcript was highly expressed. Furthermore, an *hd2c* mutant exhibited up-regulation of heat responsive genes such as *HSFA3*, *HSFC1*, *HSP101* and the ascorbate peroxidase gene *APX2* as compared to wild-type plants (Buszewicz *et al.*, 2016). Moreover, transcriptomics data from the

hd2c mutant revealed gene categories associated with heat response, protein folding and oxidative stress (Buszewicz *et al.*, 2016). Some of the other histone deacetylase genes such as *HDA9, HDA15* and *HDA19* respond differently to high temperature. The *hda9* and *hda19* mutant lines were insensitive to heat when exposed to 27°C, whereas the *hda15* mutant displayed an enhanced thermal response to the same temperature (Shen *et al.*, 2019). Rpd3-type histone deacetylase *HDA6* is hypersensitive and plays a crucial role in basal heat tolerance (Popova *et al.*, 2013). However, whether the histone modification gene *HDA6* confers stress sensitivity at the reproductive stage has not been reported. Expression of the *FvHDA6* gene is induced by heat stress in *Fragaria vesca* anthers. The fertility of *Arabidopsis* mutant *hda6* was significantly decreased under high temperature stress, suggesting that *HDA6* is required for maintaining fertility during heat stress.

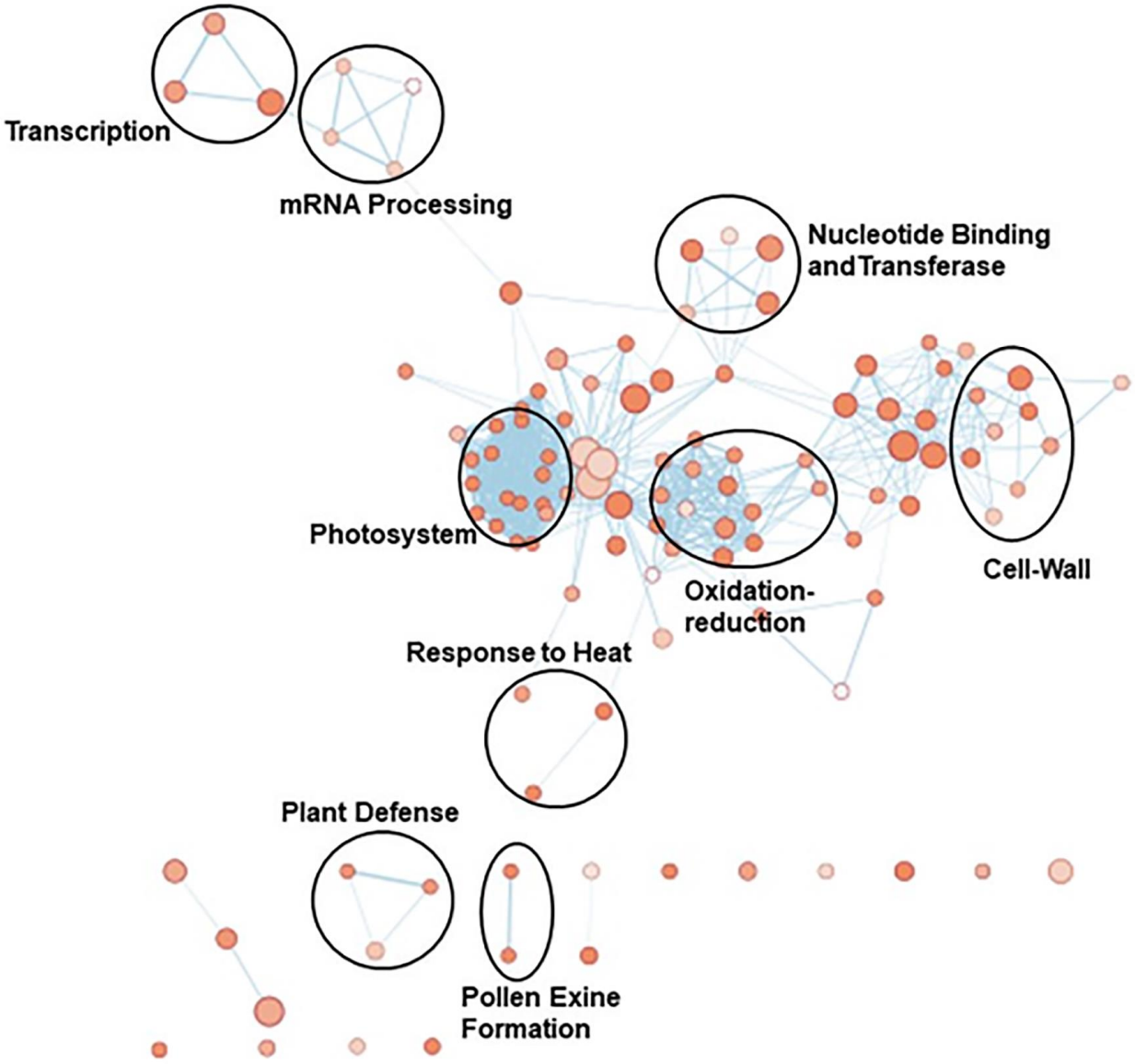

Figure 4.1 Enrichment map of Arabidopsis gene IDs converted from *Fragaria vesca* gene IDs depicting gene clusters in response to heat stress. Node size represents the gene-set size and lines indicates overlap between two different gene sets. Gene cluster sets involved in similar biological and molecular processes were manually circled and labelled.

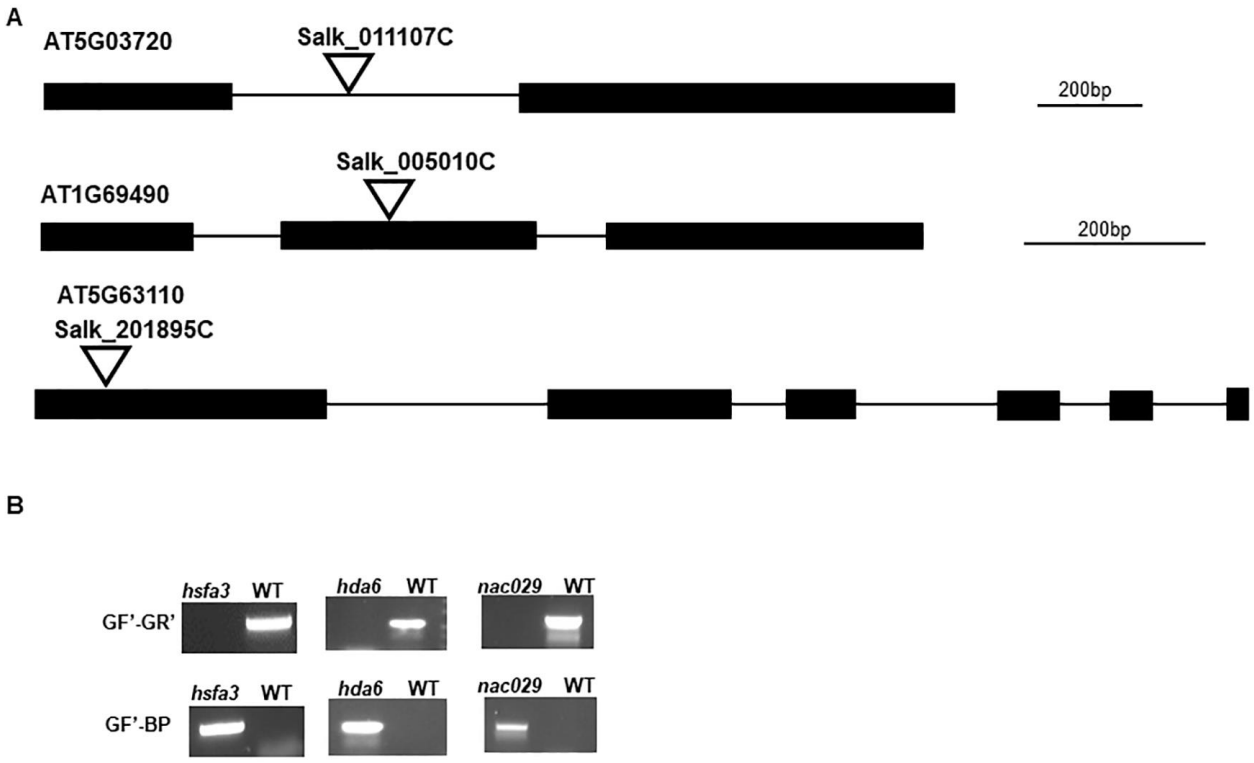

Figure 4.2 Characterization of *hsfa3, nac029,* and *hda6* T-DNA mutants of *Arabidopsis*. (A) Schematic diagram showing structures of *HSFA3* (AT5G03720), *NAC029* (AT1G69490), and *HDA6* (AT5G63110) genes and locations of T-DNA**.** Lines indicates introns and black boxes indicates exons. Empty triangles indicate T-DNA insertion sites**. (B)** PCR genotyping results of *hsfa3, nac029,* and *hda6* mutants using their respective GF'- Gene Forward and GR'-Gene Reverse primers, T-DNA insertion in lines was confirmed with GF'- Gene Forward and BP- T-DNA Border primer pairs**.** The primers used can be found in Table 4.1.

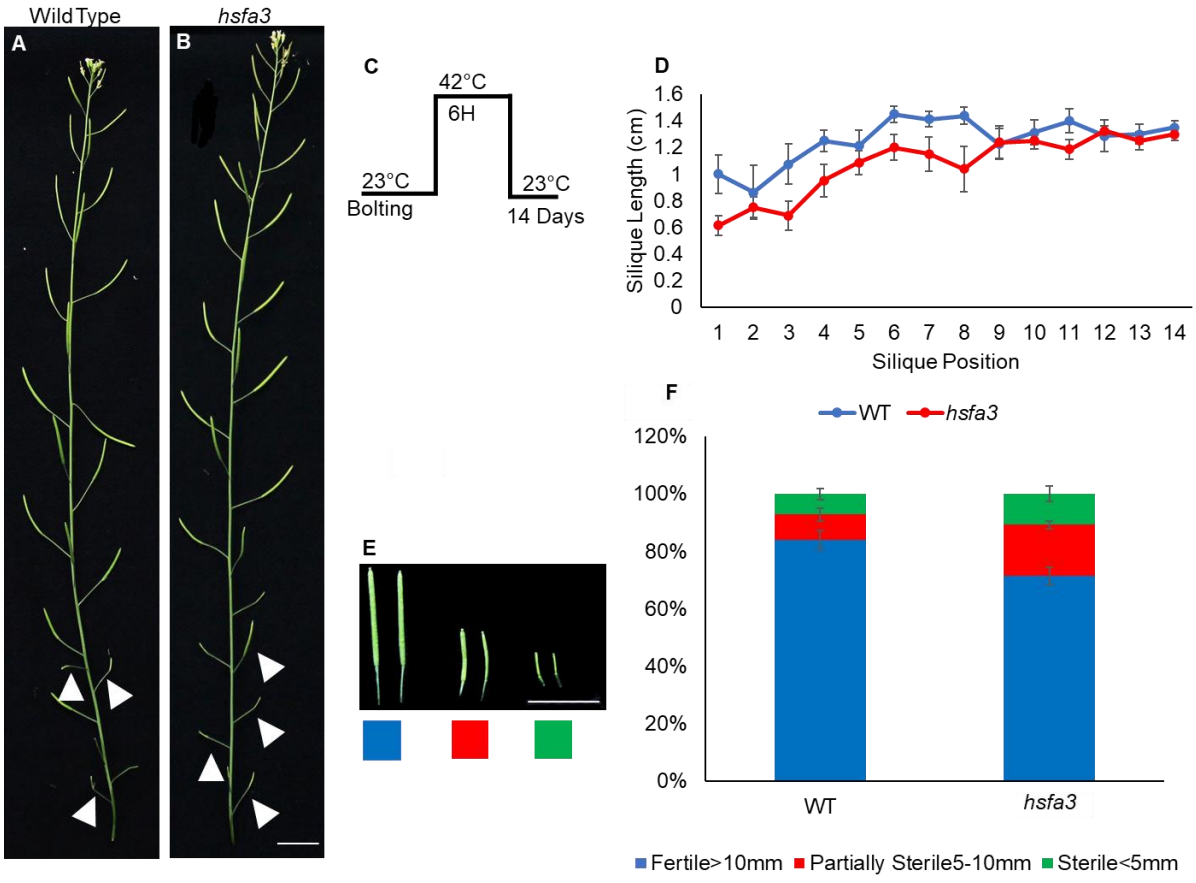

Figure 4.3 High temperature stress caused reduced fertility in the *hsfa3* mutant. (A) and **(B)** Effect of heat stress (42°C, 6 hours) on inflorescences of plants exposed at bolting stage. *Arabidopsis* wild-type (A) and *hsfa3* single mutant (B) plants. Arrow heads indicate sterile siliques. Scale bar = 1cm (A and B). **(C)** Represents the heat stress exposure and recovery cycle used in the experiment. **(D)** Silique length measured (for14 siliques) by position in each inflorescence. Phenotype was examined after 10 days of heat stress. **(E)** Siliques categorized into three types: fertile (>10mm), partially sterile (5-10mm) and sterile (<5mm) Scale bar = 1 cm. **(F)** Effect of heat stress on fertility in wild-type (WT) and *hsfa3* mutant plants.

Values represented are means ± SD, n = 8. In (D), two-way ANOVA was used for silique length and position ($p<0.0006$). In (E), one-way ANOVA was used for fertile, partially fertile, and sterile siliques. Fertile and partially sterile siliques of WT and *hsfa3* were considered statistically

significant $p<0.001$ for fertile siliques and $p<0.004$ for sterile siliques, whereas sterile siliques indicate no significant difference between WT and *hsfa3* as $p<0.2$.

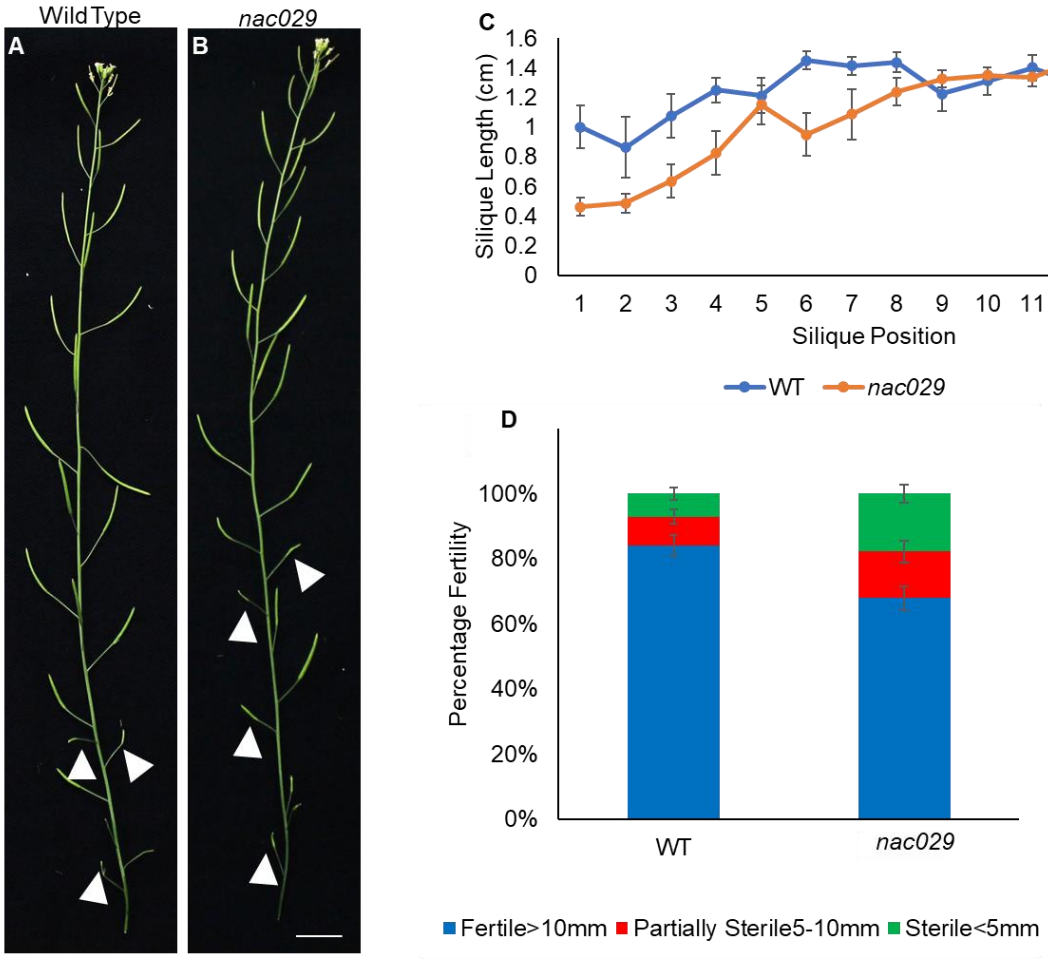

Figure 4.4 High temperature stress caused reduced fertility in the *nac029* mutant.

(A) and **(B)** Effect of heat stress (42°C, 6 hours; exposed at bolting stage) on inflorescence of wild-type (A) and *nac029* single mutant (B) plants. Arrow heads indicate sterile siliques. Scale bar = 1cm (A and B). **(C)** Silique length measured (for 14 siliques) by position in each inflorescence. Phenotype was examined after 10 days of heat stress. **(D)** Effect of heat stress on fertility of inflorescence in wild-type (WT) and *nac029* mutant plants.

Values represented are means ± SD, n = 8. In (C), two-way ANOVA was used for silique length and position ($p<0.002$). In (D), one-way ANOVA was used for fertile, partially fertile, and sterile siliques. Fertile and sterile siliques of WT and *nac029* were considered statistically significant ($p<0.004$ for fertile siliques and $p<0.005$ for sterile siliques), whereas partially sterile siliques indicate no significant difference between WT and *nac029* as $p<0.2$.

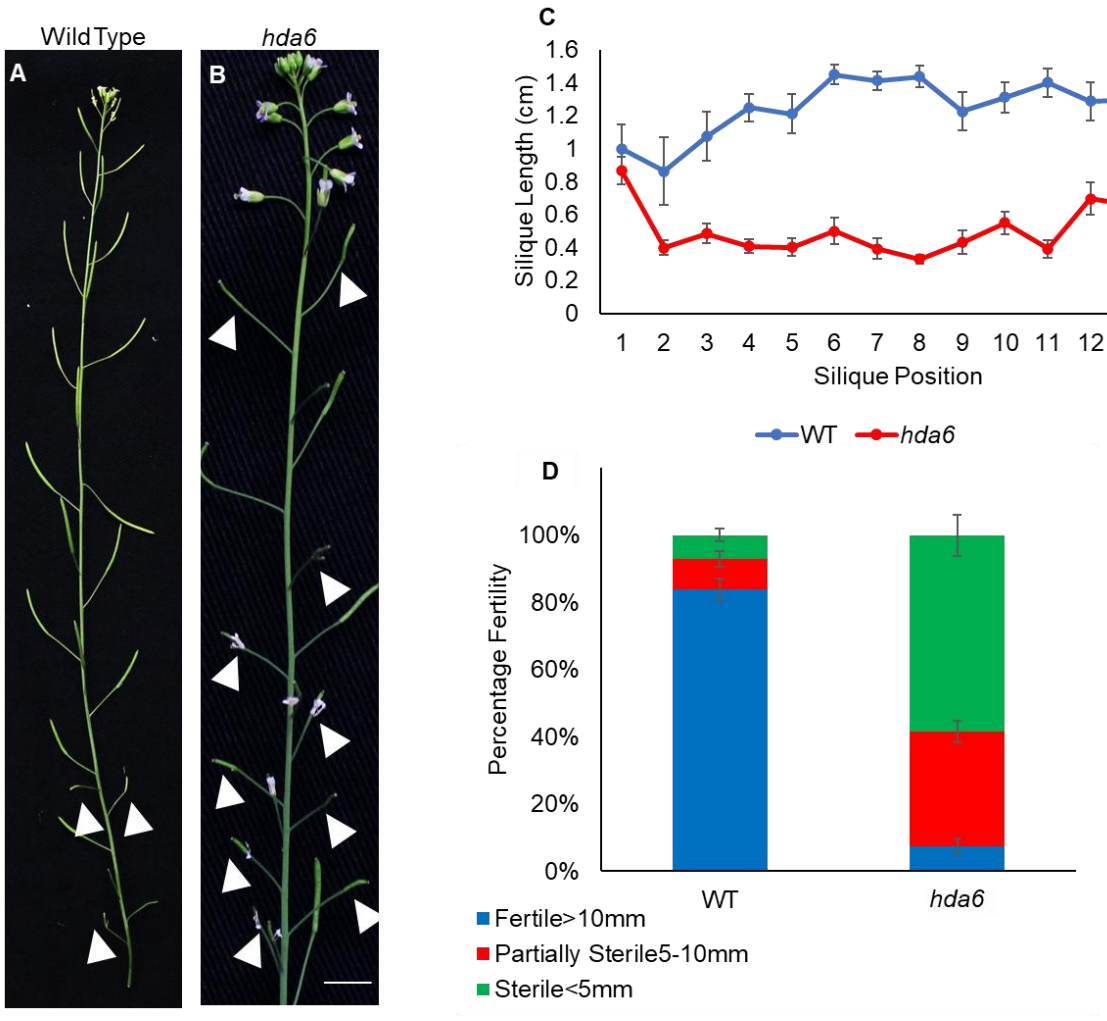

Figure 4.5 The *hda6* mutant displayed sterility upon exposure to high temperature stress. **(A)** and **(B)** Effect of heat stress (42°C, 6 hours; exposed at bolting stage) on inflorescence of *Arabidopsis* wild-type (A) and *hda6* single mutant (B) plants. Arrowheads indicate sterile siliques. Scale bar = 1cm (A and B**). (C)** Silique length measured (for14 siliques) by position in each inflorescence. Phenotype was examined after 10 days of heat stress. **(D)** Effect of heat stress on fertility in wild-type (WT) and *hda6* mutant plants. Values represented are means ± SD, n = 8. In (C), two-way ANOVA was used for silique length and position ($p<0.0001$). In (D), one-way ANOVA was used. Fertile, partially fertile, and sterile siliques were considered statistically significant when $p<0.0001$.

Tables:

Table 4.1: Primers used in this study for genotyping *Arabidopsis* mutants

Primer Name	Sequence	Purpose
SALK_201895C F	TTCTTCCACTCCATCTCCATG	*hda6*
SALK_201895C R	TGACTTTCTCAGGCATTGTTG	*hda6*
SALK_005010C F	CTTTTTAACCGTGGCTGTTTG	*nac029*
SALK_005010C R	GTCCCCGAACCAACTAGACTC	*nac029*
SALK_011107C F	AAAAGATAAATCCACGGTGGC	*hsfa3*
SALK_011107C R	AGCAAGTTTGGTTGGATTGTG	*hsfa3*

Table 4.2 List of *Fragaria vesca* genes and their orthologous genes in *Arabidopsis* for functional study

Anther Stage	**Gene Name**	***Arabidopsis* orthologue**	**Description**	**baseMean**	**log2Fold Change**	**pvalue**
Meiotic (Stage 6)						
	FvH4_6g17890	AT5G03720	Heat shock factor (HSF)-type, DNA-binding	60.739	3.434	2.7E-25
	FvH4_4g31070	AT1G69490	NAC domain	379.491	6.831	1.5E-102
	FvH4_1g25790	AT5G63110	Histone deacetylase superfamily	114.792	10.497	1.2E-18
Post-Meiotic (Stage 8)						
	FvH4_6g17890	AT5G03720	Heat shock factor (HSF)-type, DNA-binding	80.507	2.756	3.2E-28
	FvH4_4g31070	AT1G69490	NAC domain	131.572	1.696	3.5E-24
	FvH4_1g25790	AT5G63110	Histone deacetylase superfamily	134.694	1.827	3.3E-26

Table 4.3: Mean length of 14 individual siliques from wild-type and *hsfa3* plants (n=8)

WT	*hsfa3*	STDEV WT	STDEV *hsfa3*
1.000	0.613	0.145	0.072
0.862	0.750	0.204	0.078
1.075	0.688	0.150	0.109
1.250	0.950	0.082	0.122
1.212	1.088	0.120	0.090
1.450	1.200	0.060	0.096
1.413	1.150	0.058	0.127
1.438	1.038	0.065	0.171
1.225	1.238	0.116	0.122
1.313	1.250	0.093	0.060
1.400	1.188	0.089	0.074
1.288	1.325	0.117	0.037
1.300	1.250	0.076	0.065
1.350	1.300	0.050	0.046

Table 4.4: Silique fertility in wild-type and *hsfa3* plants

Plant type	Fertile>10mm	Partially Sterile5-10mm	Sterile<5mm
WT	11.750	1.250	1.000
hsfa3	10.000	2.500	1.500
WT STDEV	0.453	0.313	0.267
***hsfa3* STDEV**	0.422	0.188	0.377

STDEV = Standard Deviation

Table 4.5: Mean length of 14 individual siliques from wild-type and *nac029* plants (n=8)

WT	*nac029*	STDEV WT	STDEV *nac029*
1.000	0.463	0.145	0.060
0.863	0.488	0.204	0.064
1.075	0.635	0.150	0.112
1.250	0.825	0.082	0.149
1.213	1.150	0.120	0.131
1.450	0.950	0.060	0.144
1.413	1.088	0.058	0.169
1.438	1.238	0.065	0.092
1.225	1.325	0.116	0.059
1.313	1.350	0.093	0.050
1.400	1.338	0.089	0.062
1.288	1.450	0.117	0.042
1.300	1.450	0.076	0.042
1.350	1.388	0.050	0.044

Table 4.6: Silique fertility in wild-type and *nac029* plants

Plant type	Fertile>10mm	Partially Sterile5-10mm	Sterile<5mm
WT	11.750	1.250	1.000
nac029	9.500	2.000	2.500
WT STDEV	0.453	0.313	0.267
***nac029* STDEV**	0.500	0.462	0.377

Table 4.7: Mean length of 14 individual siliques from wild-type and *hda6* plants (n=8)

WT	*hda6*	STDEV WT	STDEV *hda6*
1.000	0.869	0.145	0.084
0.863	0.400	0.204	0.043
1.075	0.485	0.150	0.060
1.250	0.409	0.082	0.040
1.213	0.402	0.120	0.054
1.450	0.501	0.060	0.081
1.413	0.394	0.058	0.064
1.438	0.330	0.065	0.025
1.225	0.432	0.116	0.072
1.313	0.551	0.093	0.067
1.400	0.393	0.089	0.054
1.288	0.698	0.117	0.100
1.300	0.658	0.076	0.092
1.350	0.751	0.050	0.133

Table 4.8: Silique fertility in wild-type and *hda6* plants

Plant type	Fertile>10mm	Partially Sterile5-10mm	Sterile<5mm
WT	11.750	1.250	1.000
hda6	1.125	5.125	8.750
WT STDEV	0.453	0.313	0.267
***hda6* STDEV**	0.350	0.479	0.920

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Chapter 5

Summary

5.1 Heat stress affects pollen viability and fruit development in *Fragaria vesca*

Fragaria vesca, which emerges as a model system for Rosaceae, was used to study the effects of heat stress on pollen and fruit development. Heat stress exposure (at 42°C) for 2 days resulted in 60.04% pollen viability as opposed to 90.14% pollen viability under normal conditions, while 4 days of heat stress led to 100% of pollen abortion. In addition, *Fragaria vesca* plants produced malformed fruits under 42°C heat treatment for 2 and 4 days, respectively, suggesting formation of abnormal pollen is one of the main causes for aberrant fruit development under heat stress.

5.2 Heat stress causes aberrations in tapetum, microsporocytes and microspores in *Fragaria vesca*

In this work, I established a complete anther development series in *Fragaria vesca*. The anther development stages were defined on the basis of microscopic landmark events. Despite the determinate dichasial cyme inflorescence in *Fragaria vesca,* the correlation of bud size to anther stage was established to help identify key stages of anther development. Then I showed that heat stress (42°C for 2 and 4 days) affected anther development at two major stages, i.e., meiotic (stage 6) and post-meiotic (stage 8). Heat stress caused abnormal tapetum, microsporocytes, and microspores in anthers. The primary defect was hypertrophied tapetal cells occupying a majority of anther lobes. Deformed microsporocytes and microspores were also observed. I further demonstrated that prolonged exposure (i.e., 4 days) to high temperature resulted in severe damage to the anther cells than 2 days of heat exposure.

5.3 Stage-specific transcriptomics of heat stressed anthers reveals expression changes of various genes involved in heat stress response

RNA-seq was conducted on *Fragaria vesca* anthers under normal conditions and heat stress at 42°C for 2 days at meiotic (stage-6) and post-meiotic (stage-8) stages to identify genes and gene pathways in response to heat stress during anther development. I discovered that heat stress altered expression of heat stress-responsive genes including those encoding heat shock factors and heat shock proteins, as well as genes involved cell wall formation, oxidation-reduction, and transcriptional regulation. Moreover, histone modification genes were also mis-regulated during heat stress, suggesting epigenetic regulation plays an important role in heat stress response during anther development. Furthermore, anthers at the meiotic stage appear to be more active in gene transcription than at the post-meiotic stage.

5.4 Functional analysis of putative heat responsive genes in heat stress response

The potential function of some key genes identified by RNA-seq analysis in heat stress were examined using the model plant *Arabidopsis*. It was observed that some genes from transcription factor and histone modification categories were highly regulated during heat stress. Therefore, three genes, *HEAT SHOCK FACTOR A3, NAC029* and *HISTONE DEACETYLASE 6,* were chosen to test their function in *Arabidopsis*. The results demonstrated that all three mutants, namely *hsfa3, nac029*, and *hda6,* were sensitive to heat stress. The percentages of partially sterile and sterile siliques were higher in all three heat-treated mutants than that in the wild type. Moreover, 62.5% sterile siliques were observed in the *hda6* mutant, which was highest among *nac029, hsfa3* and wild-type plants. These mutant plants are normal under normal condition, suggesting that their role in heat stress is specific to reproductive development.

5.5 Significance of this work

The current work contributes towards the study of fertility defects caused by heat stress in fruit crops, especially strawberry. This also lays the groundwork for establishing an anther developmental series with defined cellular landmarks for the Rosaceae family. Moreover, profiling of gene expression in heat stressed anthers at two key stages provides insight into major gene pathways in response to heat stress.

Under normal conditions, pollen development is followed by subsequent fertilization leading to fruit formation (Fig. 5.1). High temperature causes defects in tapetum, microsporocytes, and microspores that affect pollen development. Abnormal pollen leads to defects in fertilization and consequently the formation of deformed fruits. I propose a molecular mechanism that involves differential regulation of transcription factors and histone modification gene that lead to heat stress response in anther (Fig. 5.1). In the working model, transcription factors such as Heat Shock Factor A3 and NAC029 could activate expression of heat stress responsive genes to achieve thermotolerance during anther development. On the other hand, epigenetic control via HDA6 mediated histone modification might be critical for plants to manage heat stress during anther development. My work provides a basis for further investigating the molecular mechanisms underlying heat stress during male reproduction development. Moreover, my results could be important for creating thermotolerant varieties to withstand high temperatures.

5.6 Future work on *Fragaria vesca* heat stress

I observed partial and complete male sterility in *Fragaria vesca* plants following 2- and 4-days of 42°C heat stress, respectively. I then found that heat stress caused major anomalies in tapetal

cells, microsporocytes, microspores, and pollen grains. To further support the results obtained by semi-thin sections, transmission electron microscopy can be utilized in future research for studying the ultrastructural details of anther cells during heat stress, specifically tapetum and microspores during heat stress.

Moreover, it will be worthwhile to test the effect of mild heat (e.g., temperature range from 32-38°C) on anther development. To support this, I would section heat-treated anthers at meiotic and post-meiotic stages and compare the results with the established anther series.

Some representative genes identified from the RNA-seq analysis were chosen for testing their function in heat stress using *Arabidopsis* due to the unavailability of *Fragaria vesca* mutants. The preliminary data suggests involvement of heat shock factor and an NAC transcription factor in thermotolerance during reproduction. It is needed to examine whether male or female reproductive organs are responsible for this silique sterility. To establish male organ involvement, I can perform Alexander staining to examine pollen grain viability. Additionally, to establish female reproductive organ involvement, I can pollinate the heat-treated carpels using normal untreated pollen. I could also perform floral tissue sectioning that can help in identifying key anther development stages sensitive to heat stress.

Additionally, histone modification genes were also found critical for heat stress during anther development. Identification of the association of histone modification genes with heat stress opens a new avenue to study epigenetic control in heat stress. Histone covalent modifications play a vital role in chromatin structure and remodeling that may regulate acclimation to heat stress. One of the histone modification genes known as *hda6* has been shown to be hypersensitive to heat stress at the vegetative phase (Popova *et al.*, 2013). Therefore, more work needs to be performed to study its role during heat stress during male reproduction. Future

research can include the generation of overexpression lines with the constitutively active promoter *35S CaMV*, which can help in the study of the *HDA6* function during heat stress. Firstly, semi-thin sectioning can identify anther stages affected due to heat stress. Secondly, to better understand the role of histone modification genes such as histone deacetylase, I can perform *in situ* hybridization to show the expression pattern of *HDA6* in anthers. Thirdly, genes and gene networks associated with *HDA6* via transcriptomics analysis can be studied.

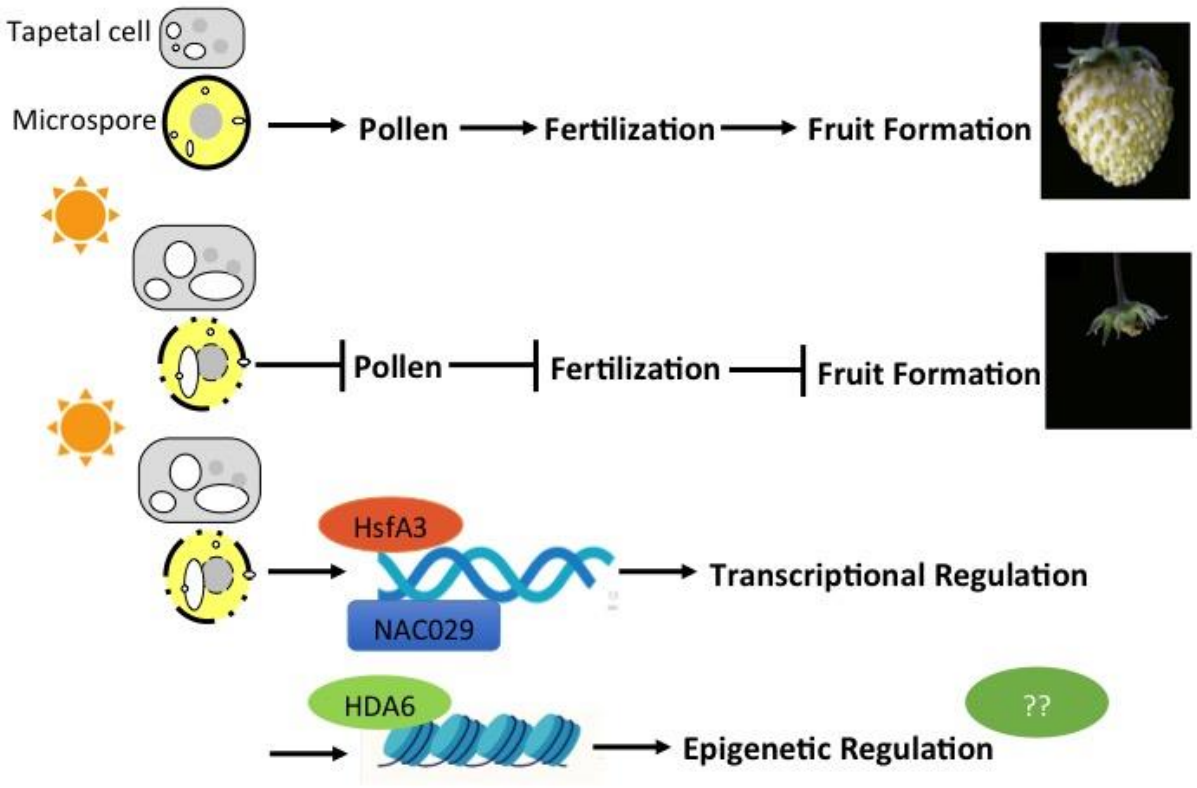

Figure 5.1 Schematic diagram showing defects in male reproduction and working models of gene regulation under heat stress. Defects in tapetal cells and microspores as a consequence of heat stress leads to disruption in pollen formation followed by deformities in fruit formation. Heat stress induces expression of *HSFA3* and *NAC029*, which encode transcription factors regulating expression of heat responsive genes. Moreover, the histone deacetylase 6 (HDA6) also plays an important role in heat stress response via epigenetic regulation. Arrows denotes induction and T-bars represent inhibition.

References

Popova, O.V., Dinh, H.Q., Aufsatz, W., and Jonak, C. (2013). The RdDM pathway is required for basal heat tolerance in *Arabidopsis*. **Molecular Plant** *6,* 396-410.

Curriculum Vitae

Shikha Malik

EDUCATION

- **PhD: Cell and Molecular Biology** (Aug 2014-Dec2020)
  Department of Biological Sciences, University of Wisconsin-Milwaukee, USA)

- **Master of Science: Botany (Honors)** (Jul 2010 – May 2012)
  Department of Botany, University of Delhi, India

- **Bachelor of Science (Honors): Biomedical Sciences** **(**Jul 2007 – May 2010)
  Sri Venkateswara College, University of Delhi, India

PEER-REVIEWED PUBLICATIONS

1. Huang J, Li Z, Biener G, Xiong E; **Malik S**, Eaton N, Zhao C, Raicu V, Kong H, Zhao D. Carbonic Anhydrases Function in Anther Cell Differentiation Downstream of the Receptor-Like Kinase EMS1. **Plant Cell**

AWARDS AND SCHOLARSHIPS

1. *Chancellors Graduate Student Award,* Department of Biological Sciences, University of Wisconsin, Milwaukee. August 2014 – 2019.
2. *Ruth I Walker – Graduate Grant-in-Aid Award,* Department of Biological Sciences, University of Wisconsin, Milwaukee. April 2018.
3. *Travel Award,* Department of Biological Sciences, University of Wisconsin, Milwaukee. June 2017.
4. *Midwest Plant Cell Dynamics Award,* Midwest Plant Cell Dynamics, Madison. May 2017.
5. *Raymond E. Hatcher Memorial Scholarship,* Department of Biological Sciences, University of Wisconsin, Milwaukee. April 2016.
6. *Best Poster Award, Biological Sciences Research Symposium,* Department of Biological Sciences, University of Wisconsin, Milwaukee. April 2016.
7. *Merit for 1st position,* Sri Venkateswara College M.Sc. Botany- Final Year. 2011-2012.
8. *Mrs. P. Gupta Meritorious prize for 1st position,* Sri Venkateswara College B.Sc. Botany (Hons.)- 3rdyear. 2010-2011.
9. *Bharatula Savitri Memorial prize for 1st position,* Sri Venkateswara College B.Sc. Botany (Hons.)- 3rdyear.2010-2011.
10. *Smt. Narayanan Memorial Medal for 1st position,* Sri Venkateswara College B.Sc. Botany (Hons.)-3rd year.2010-2011.
11. *Merit prize 1st position,* Sri Venkateswara College B.Sc. Botany (Hons.). 2010.
12. *Merit prize 1st position,* Sri Venkateswara College B.Sc. Botany (Hons.). 2009

ABSTRACTS (presenting author is underlined) PRESENTATION AND POSTERS

1. **Malik S.** Morphological and transcriptomics analysis of heat stress on anther development in *Fragaria vesca*. Colloquium, University of Wisconsin, Milwaukee (November 2019).
2. **Malik S**, Zhao D. Effect of heat stress on anther development in *Fragaria vesca*, Biological Science Symposium, University of Wisconsin, Milwaukee (April 2018).
3. **Malik S**, Zhao D. Effect of heat stress on anther development in *Fragaria vesca*, Biological Science Symposium, University of Wisconsin, Milwaukee (April 2017).
4. Huang J, Li Z, Biener G, Xiong E; **Malik S**, Eaton N, Zhao C, Raicu V, Kong Zhao D. Carbonic Anhydrases Function in Anther Cell Differentiation Downstream of the Receptor- Like Kinase EMS1. International Conference on Arabidopsis Research (ICAR), Missouri, USA (June 2017).
5. **Malik S**, Zhao D. Effect of heat stress on anther development in *Fragaria vesca*, Midwest Plant Cell Dynamics, Madison, Wisconsin, USA (June 2017).
6. **Malik S**, Zhao D. Exploring the Function of β- Carbonic Anhydrase gene family in Abscisic Acid (ABA) Signaling Cascade, Biological Science Symposium, University of Wisconsin, Milwaukee (April 2016).

SUPERVISION OF STUDENTS

As a Ph.D. student, I was given the opportunity to supervise the following students:

1. Undergraduate student: Christian Michael Umanos (Jan 2018 – Jan 2019)
2. Undergraduate student: Jamal Hassan (Aug 2018 – Dec 2018)
3. Undergraduate student: Ryan (Sep 2016 – Dec 2018)

LEADERSHIP, VOLUNTEERING AND PARTICIPATION

1. Lead Teaching Assistant Anatomy and Physiology: 2017- Present
2. Symposium organizer, Biological Sciences annual symposium 2018, Department of Biological Sciences, University of Wisconsin, Milwaukee.
3. Symposium organizer, Biological Sciences annual symposium 2017, Department of Biological Sciences, University of Wisconsin, Milwaukee.
4. Student co-organizer; Graduate research symposium for Math, Engineering and Natural sciences October 2016
5. Vice-President; Graduate Organization Biological Sciences 2018
6. Office bearer, Graduate Organization Biological Sciences 2017