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A Cell Atlas of Rice Anther Development under Heat Stress

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Abstract Rice anther development is a crucial determinant of male fertility and overall grain yield, yet it is particularly sensitive to elevated temperatures that threaten global rice productivity. This study constructed a comprehensive single-cell atlas of rice anther development under heat stress, integrating single-cell RNA sequencing, spatial transcriptomics, and multi-omics analyses to reveal cell type-specific responses to thermal conditions. The results demonstrate that heat stress disrupts tapetal function, alters pollen fertility, and reprograms transcriptomic profiles across key somatic and germline layers, with pronounced effects on reactive oxygen species signaling, hormone pathways, and programmed cell death regulation. Through a detailed case study of the tapetum, this study identified transcriptional networks and resilience mechanisms contributing to thermal tolerance at the cellular level. This work provides a high-resolution framework for understanding the molecular and cellular bases of heat-induced reproductive failure in rice and offers valuable genetic markers and targets for breeding heat-resilient varieties, paving the way for precision breeding under changing climatic conditions.

Keywords Rice anther development; Heat stress; Single-cell RNA sequencing; Tapetal cell resilience; Precision breeding

1 Introduction

Worldwide, approximately half of the population takes rice (*Oryza sativa* L.) as their main food source. Whether rice can reproduce successfully depends on whether the male organs, especially the anthers, can develop normally. The development of anthers is not a simple process. It goes through multiple stages such as cell differentiation, meiosis and pollen maturation, and each step requires meticulous coordination. Only when all these steps proceed normally can fertile pollen be formed and fertilization be completed (Hu et al., 2020). Interestingly, the structure of anthers is extremely dynamic, and the meiosis and microspore development stages are particularly susceptible to environmental interference. In recent years, studies in the transcriptome and proteome have enabled us to see the complex networks regulating andel development and reveal the key role of post-translational modifications in it. All these indicate the importance of precise molecular regulation for the reproductive capacity of rice (Liu et al., 2020).

However, natural conditions are not always ideal. Global warming has made heat stress events more frequent and intense, and the impact on rice production is becoming increasingly difficult to ignore. Even a short period of high temperature during the flowering period may inactivate pollen, impede spikelets' development, and eventually lead to reduced yields (Shrestha et al., 2022). High temperatures can disrupt the normal physiology of anthers, such as the cracking process, maintenance of pollen vitality, and carbohydrate metabolism. Worse still, it can also induce premature degradation of the felt-like layer, disrupt pollen wall formation, and trigger excessive accumulation of reactive oxygen species (ROS) (Guan et al., 2023; Zhao et al., 2023b). Not all varieties are equally fragile. Heat-tolerant genotypes tend to maintain a more stable anther structure, more efficient sugar utilization capacity, and stronger protective protein expression (Kumar et al., 2023; Mo et al., 2023).

Based on this, this study aims to integrate the existing research results on the cellular and molecular responses of anthers at high temperatures and establish a comprehensive map that combines morphological, transcriptomic, proteomic and metabolomic data. By comparing the differences between heat-tolerant and heat-sensitive rice, we

attempt to reveal the cellular mechanisms and molecular pathways of reproductive heat tolerance. The ultimate goal is not merely theoretical understanding, but to provide a basis for future molecular improvement and breeding, helping to cultivate rice varieties that can maintain high yields in warming climates.

2 Overview of Rice Anther Cell Types

2.1 Key somatic and germline cell layers

The basic structure of rice anolls is not complex, but the internal layers are clear: the epidermis, endocortex, mesosphere and felt-like layer are almost like four nested cells, surrounding the pollen mother cells (PMCs) located in the center (Araki et al., 2020). The responsibilities of these layers are not the same. The epidermis mainly serves a protective function. The endodermis affects whether the anthers can crack smoothly in the later stage. The middle layer will gradually degenerate as the pollen matures. The innermost felt-like layer is responsible for providing nutrients to the developing pollen, participating in the formation of the pollen wall, and entering programmed cell death (PCD) at the appropriate stage.

However, the early cellular fate of anthers is not spontaneously determined. Signaling molecules such as receptor-like kinase MSP1 and its ligand OsTDL1A need to be "laid a foundation" in advance to balance the differentiation between somatic cells and germ cells. And genes like *MIL2* and *TIP2* further intervene to ensure that each layer can form according to the established pattern; otherwise, both the structure and function of the anthers will be affected.

2.2 Transcriptomic signatures of individual cell types

Although both the felted layer and microspores originate from the L2 layer, the gene expression patterns presented by cell type-specific transcriptome analyses (such as laser microdissection techniques) are completely different. A large number of transcripts related to secondary metabolism, fatty acid synthesis, protein secretion and gibberellin signaling can often be seen in the felt-like layer, which is in line with its function in supporting pollen development (Ye et al., 2024). In contrast, the microspore stage places more emphasis on genes related to the cytoskeleton, cell wall remodeling and pollen tube growth, all of which are associated with pollen maturation and subsequent germination.

It is worth noting that the felt layer and microspores may show synchronous gene expression at certain times, suggesting that there may be a synergistic regulation between the two. In addition, small RNA pathways also show significant cell type differences. For example, miR2118-dependent phasiRNA is enriched in the anther wall, while the types and contents of phasiRNA in germ cells are completely different (Tamotsu et al., 2023).

2.3 Spatial and temporal dynamics during development

Anthers gradually progress from prospore cells to meiosis and then to pollen maturation. During this process, corresponding structural and molecular changes occur in each layer of cells (Araki et al., 2022). Three-dimensional imaging and immunostaining techniques help researchers more clearly observe these changes, such as the localization and transformation of proteins at different stages and the fluctuations in transcript abundance (Figure 1).

Among these processes, communication between somatic cells and germ cells is extremely crucial. Before and after the initiation of meiosis, the relevant signals need to be consistent between the two; otherwise, developmental deviations will occur (Zhao et al., 2021). The timing of the felt-like layer is particularly important: its programmed cell death, lipid metabolism adjustment, and REDOX state control will directly affect whether pollen can develop normally and ultimately be reproducible (Shi et al., 2024).

3 Molecular Effects of Heat Stress on Anther Cells

3.1 Disruption of tapetal function and pollen fertility

During the flowering period of rice, high temperatures often first affect the velvety layer. It should enter programmed cell death (PCD) at a specific rhythm to provide matter and space for pollen. However, under heat stress, this process often becomes disordered, and ROS accumulation and nutrient supply interruption also occur

(Lin et al., 2023). The result is a decline in pollen vitality, anthers are not easy to crack, and the fertility of spikelets is significantly impaired. Of course, not all varieties of felt layers are equally fragile. Sensitive varieties tend to show excessive degeneration earlier, with higher levels of ROS and ABA, and more severe cell membrane damage. Heat-resistant varieties can maintain a relatively stable structure. These differences once again indicate that the felt-like layer plays a key role in heat-induced male sterility.

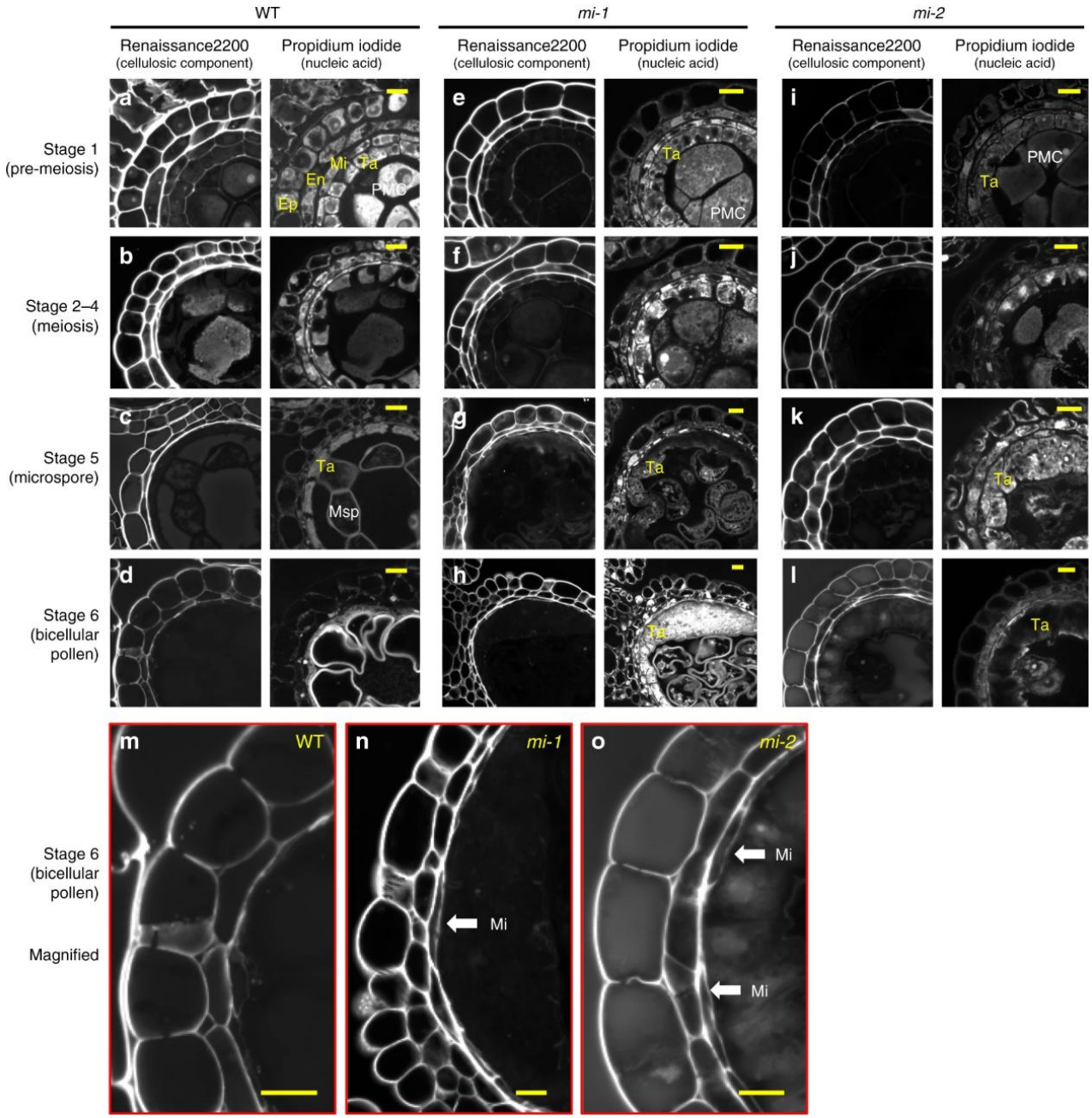

Figure 1 miR2118 is involved in the maturation of the anther wall during post meiosis

Image caption: a-l Cross-section images of anthers of WT (a-d), *mi-1* (e-h), and *mi-2* (i-l). Panels depict anthers that were stained with Renaissance 2200 (left) and propidium iodide (right). Stage 1 is pre-meiosis, or primordial germ-cell initiation (a, e, and i); Stages 2-4 are during meiosis (b, f, and j); Stage 5 is the microspore development stage (c, g, and k); and Stage 6 is the bicellular pollen stage (d, h, and l). Tapetum defects were observed in *mi-1* and *mi-2* at Stages 5 and 6 after meiosis (g, h, k, and l). m-o Enlarged images of the anther wall that was stained with Renaissance 2200 of d, h, and l. Middle layers were retained at later stages, even at Stage 6, in both *mi-1* and *mi-2* compared to the Mi-collapse in the WT anther wall at Stage 5. Ep: Epidermis, En: endothecium, Mi: middle layer, Ta: tapetum, PMC: pollen mother cell, Msp: microspore. The white arrows indicate Mi. Yellow bars represent 10 μm

3.2 Transcriptomic reprogramming under heat

Under high-temperature conditions, the gene expression of anther cells changes rapidly, which can be clearly seen from transcriptome and proteome analyses. The expression of heat shock proteins (HSP) and molecular chaperones usually increases significantly. They are responsible for mitigating the effects caused by protein misfolding (He et al., 2023). Meanwhile, genes related to antioxidant enzymes will also be activated to deal with the accumulated ROS.

After comparing different genotypes, it can be found that heat-resistant materials can better maintain higher levels of non-structural carbohydrates, ATP and antioxidants, while sensitive varieties have more chaotic responses in terms of carbon metabolism, hormone signals (especially ABA and GA), etc. (Zhou et al., 2022). In addition, the expression changes of different transcription factor families, including HSF, NAC, AP2/ERF, WRKY, and MYB, at high temperatures also indicate that the regulatory network has been widely reprogrammed in a short period of time.

3.3 Comparative analysis with normal conditions

If the anthers under heat stress are compared with those under normal temperature conditions, it will be easier to clearly see the differences between heat-resistant and sensitive varieties. Heat-resistant materials can often maintain the integrity of anther structure, high antioxidant enzyme activity and stable energy state, while sensitive varieties are more prone to premature degradation of the felty layer, decreased pollen viability, and a series of dysregulated transcriptional and metabolic reactions (Guo et al., 2024).

These differences will eventually be reflected in fertility and yield. Especially the upregulation ability of protective genes and the ability to maintain ROS homeostasis often become the most direct indicators to distinguish between two types of varieties, and also determine which can maintain yield and which will suffer significant losses under high-temperature conditions.

4 Tools and Technologies for Building the Cell Atlas

4.1 Single-cell RNA sequencing (scRNA-seq)

When studying the anthers of rice, many cell types are often mixed together and difficult to distinguish. However, scRNA-seq is precisely designed to address this "visible but inseparable" issue. It can read the gene expression of each single cell, making the differences and developmental trajectories between cells clearer. Previous studies have applied this technology to different tissues of rice, not only identifying multiple cell types, but also observing that they would produce their own specific expression responses under stress such as high temperature (Zong et al., 2022).

This technology can also reconstruct the cell development path and even identify some cells that are scarce or in the transition period. Coupled with the combination of single-cell epigenetics and chromatin accessibility analysis, researchers were able to depict the regulatory network more accurately at the cellular level (Wang et al., 2021).

4.2 Spatial transcriptomics and in situ hybridization

If RNA-SEq resolves the question of "who is who", then spatial transcriptomics is more like answering "Where is it". This technology can map the distribution of gene expression in an entire tissue section without damaging the tissue structure, thus preserving spatial information.

As for verifying the expression sites of specific genes, in situ hybridization (ISH) remains the most commonly used and reliable method. Today's ISH processes are capable of detecting both small Rnas and micrornas, which are crucial in anther development and stress responses (Koizumi and Komiya, 2022). Some new spatial transcriptome platforms can even achieve near-single-cell resolution, enabling high-precision localization of the entire transcriptome (Su et al., 2021; Robles-Remacho et al., 2023).

4.3 Multi-omics and imaging approaches

To depict the state of cells, relying solely on one omics is often insufficient. Therefore, data from single-cell

transcriptomes, epigenomes, proteomes, and metabolomes often need to be integrated together (Wu et al., 2024). Computational frameworks like GLUE can connect this information to help correct cell type annotations and construct more complete regulatory networks (Cao and Gao, 2022; Baysoy et al., 2023).

Beyond molecular data, high-resolution three-dimensional imaging and traditional histology can reveal the specific structure and cell arrangement of anthers, while laser microdissection can separate specific cell types for further analysis. The combination of multiple techniques constitutes the core tool for studying the development process of rice anthers under heat stress, providing the possibility for establishing detailed cell maps.

5 Heat Stress-Responsive Pathways in Rice Anthers

5.1 Heat shock proteins and transcription factors

In anthers, when high temperatures come, the first to respond are often heat shock proteins (HSP) and heat shock transcription factors (HSF). These molecules are mainly responsible for maintaining protein folding and cellular homeostasis. Genes like *HSFA2a*, *HSFA2d* and *HSFA4d* all significantly enhance their expression at high temperatures, thereby driving the upregulation of a series of HSPS and improving the heat resistance of anthers.

However, regulation does not rely solely on HSF. Families such as AP2/ERF, bZIP, NAC, MYB, and WRKY will also get involved. For example, ERF74/77/108/125 can directly activate HsfA2c, while OsbZIP14 and MYB61 are involved in regulating more gene networks related to metabolic adaptation (Chen et al., 2022). In addition, the WRKY10-VQ8 module and NAC heterodimer also regulate ROS balance, allergic reactions and grain filling at high temperatures (Ren et al., 2021).

5.2 ROS, hormone, and PCD signaling

High temperatures can cause a sharp increase in ROS in anthers. Once ROS accumulates excessively, the felt-like layer can easily enter programmed cell death (PCD) prematurely, and pollen will subsequently lose its activity (Xu et al., 2023). To avoid this situation, plants rely on antioxidant enzyme systems such as APX, CAT, and SOD, as well as flavonoid pathways regulated by factors like MYB61 and UGT706F1 to eliminate ROS.

Hormones also play a key role in it. At high temperatures, ABA tends to rise rapidly. On the one hand, it promotes the outbreak of ROS; on the other hand, it accelerates the early PCD of the felt layer. In contrast, ethylene signaling tends to enhance antioxidant capacity while increasing the expression of HSF, thereby alleviating stress to a certain extent. The relationship among ROS, hormones and PCD is not linear but rather mutually restrictive and jointly determining whether anthers can develop smoothly.

5.3 Epigenetic and post-transcriptional regulation

In thermal responses, many key regulations are not directly derived from the genes themselves but are accomplished at the epigenetic level, including DNA methylation and small RNA pathways. OsAGO2 is a case in point. It inhibits OsHXK1 through epigenetic means, thereby influencing the generation of ROS and the timing of PCD in the felt layer, which is directly related to whether pollen can maintain its vitality.

In addition, high temperature can also change the splicing mode of genes. For example, *OsHSFA2d* and *OsbZIP50* exhibit selective splicing at high temperature, initiating the unfolded protein response (UPR) pathway to restore intracellular protein homeostasis (Qiu et al., 2023). Post-transcriptional regulation is also very important. For instance, the mRNA degradation and processing body formation involved in *OsCAF1A* will further refine the speed and amplitude of thermal response. The combined effect of multiple regulations enables the anthers of rice to maintain relatively flexible and precise response capabilities at high temperatures.

6 Case Study: Cell-Resolved Heat Response in Tapetum

6.1 Experimental model and methodology

When studying the response of felt layers to high temperatures, it is usually not done by relying on just one method, but by combining physiological, molecular biological and genetic approaches. Experimental materials are generally selected from both heat-sensitive and heat-tolerant rice varieties. Sometimes, some mutants related to hormone signaling or programmed cell death (PCD) are also added.

Heat treatment is mostly scheduled during the critical period of anther development, followed by histological observation, Caspase 3 activity detection, and expression analysis of important genes in the felted layer (such as *EAT1*, *MIL2*, and *DTM1*). If it is necessary to further clarify the regulatory pathways, attempts will also be made to apply exogenous salicylic acid or abscisic acid, or to use inhibitors to interfere with certain signaling processes, in order to help understand the reaction mechanism of the felt layer at high temperatures.

6.2 Tapetal cell fate alteration under heat

Under high-temperature conditions, the most common problem that occurs in the felt layer is the premature initiation or even excessive occurrence of programmed cell death (PCD). This will cause the felty layer to deteriorate more quickly than normal, and the pollen will also lose its activity as a result. The increase of ABA accompanied by high temperature usually triggers the concentrated outbreak of ROS, further promoting the PCD of the felt layer (Zhao et al., 2023a).

The activity of Caspase 3 is a commonly used marker, which significantly increases in anthers after heat treatment, especially when no protective measures are taken. Genes related to the development of the felt-like layer (*EAT1*, *MIL2*, *DTM1*) are also inhibited under such conditions, which affects nutrient supply and pollen formation, ultimately manifesting as decreased fertility and low seed setting rate.

6.3 Insights into tapetal resilience mechanisms

Whether the felt layer can recover after high temperature is largely related to whether the regulation of ROS and hormone signals is in place. Exogenous application of salicylic acid (SA) can often reduce the accumulation of ROS, prevent premature occurrence of PCD, and re-express key developmental genes, thereby increasing pollen viability and seed setting rate (Figure 2) (Feng et al., 2018).

On the other hand, the regulation at the genetic level cannot be ignored either. For instance, SAPK2 is involved in ABA signal transduction. Mutations lacking SAPK2 will show significant abnormalities in ROS dynamics and PCD responses, indicating that precise hormone regulation is crucial for maintaining the stability of the felt-like layer. Overall, whether through genetic pathways or chemical intervention, as long as ROS and hormone signals can be balanced, there is a chance to enhance the heat tolerance and reproductive performance of the rice felt-like layer.

7 Implications for Breeding Heat-Resilient Rice

7.1 Candidate genes and markers for selection

In recent years, people have accumulated a considerable amount of information on QTL and candidate genes regarding the heat tolerance of rice, especially during the reproductive period. Through high-resolution localization and meta-analysis, some stably existing QTLS were localized to the small segments of chromosomes 3, 5, 8, 9 and 12, which are small enough to be directly used for molecular marker-assisted selection (Ravikiran et al., 2022).

Among the numerous candidate genes, heat shock proteins (OsBiP2, OsMed37_1, HSP20/α -lens protein family) are frequently mentioned. Some transcription factors (OsWRKY10, OsWRKY21, OsVQ30) also frequently appear in heat tolerance studies, and some genes are involved in glucose metabolism or stress signal transduction. SSR markers such as RM6100, RM236, RM337, RM5749, and a batch of SNP markers have been proven to be associated with spikicle seed setting rate and yield under high temperature conditions (Waghmare et al., 2021; Stephen et al., 2023, is very practical in breeding.

7.2 Integration with phenotyping and field data

Molecular markers alone are not enough. To truly pick out heat-resistant materials, genomic data and phenotypic evaluation must be combined (Zhang et al., 2024). In order to enable different varieties to flower at approximately the same time, controlled environmental facilities or batch sowing are commonly used in experiments. Only in this way can key traits such as spikelet setting rate and yield be accurately measured (Visakh et al., 2024).

GWAS or transcriptome studies conducted under field conditions have also continuously discovered new QTLS and can verify candidate genes in different genetic backgrounds. The heat tolerance gene pool can be further expanded by using imported lines, local varieties or wild relatives for hybridization (Stephen et al., 2022). Ultimately, it is still necessary to link the markers with reliable field phenotypes to ensure that the selected strains remain stable under real high-temperature conditions.

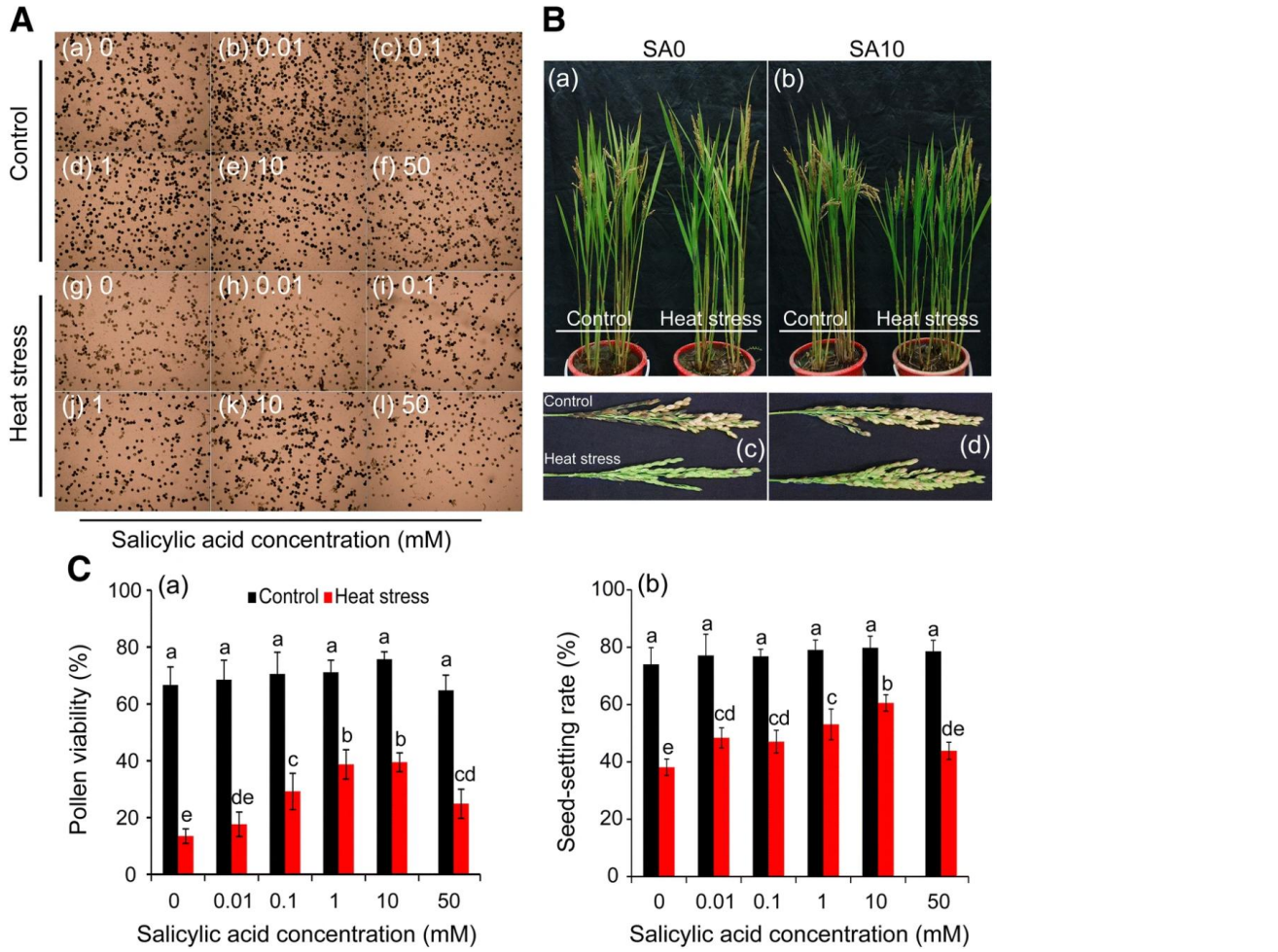

Figure 2 Effect of SA on the pollen viability and seed-setting rate of rice under heat stress at the pollen mother cell meiosis stage. a, the images of pollen grains in rice plants sprayed with salicylic acid under control and heat stress; a-f and g-i were the images of pollen grains of rice plants under control and heat stress, respectively. b, the images of rice plants with panicles sprayed with SA under control and heat stress. a and c, the images of rice plant sprayed with H2O (SA0); b and d, the images of rice plants and panicles sprayed with 10 mM SA (SA10). c, the data in the figure (a) and (b) were shown as the mean of ten and three replicates, respectively. Vertical bars denote standard deviations ($n = 10$ and 3 in (a) and (b), respectively). Different letters indicate significant differences between the SA treatments under control and heat stress ($P < 0.05$)

7.3 Future prospects in precision breeding

In the future, in terms of heat-tolerant breeding, multi-omics tools (genomics, transcriptomics, proteomics) are likely to play an increasingly significant role, along with big data analysis and more advanced phenotypic platforms (Raza et al., 2020). CRISPR/Cas9 editing, genomic selection and high-throughput genotyping techniques can all significantly accelerate the breeding of new heat-resistant varieties.

Building a germplasm bank containing known heat-resistant materials and using validated markers during the MAS process can help introduce favorable alleles into the target strain as soon as possible (Zhou et al., 2024). Meanwhile, continuous functional verification of candidate genes and their regulatory regions, as well as final confirmation in the field, will also be a key link in maintaining the heat tolerance of rice in the future.

8 Concluding Remarks and Future Directions

In recent years, with the continuous development of cell resolution technology and multi-omics analysis, people have gained more detailed observations on the response of rice anthers at high temperatures than ever before. Some core points that repeatedly emerged in the research have gradually become clear, such as heat shock proteins, transcription factors, and metabolic pathways related to non-structural carbohydrates and ATP, which have a significant impact on the heat tolerance of anthers and pollen. Heat-resistant genotypes usually can maintain a relatively intact anther structure at high temperatures, have higher antioxidant enzyme activity, and more stable sugar metabolism. These characteristics will eventually be reflected in pollen vitality and spikelets setting rate. Meanwhile, the balance changes of hormones such as ABA and GA, as well as the management methods of ROS, also largely determine the fate of the felted layer and the success or failure of reproduction.

However, these understandings are still not complete. There are currently no particularly clear models for some issues, such as how carbon metabolism, hormone signaling and ROS homeostasis interact in different anther cell types. The dynamic relationships among the transcriptome, post-transcriptional regulation and proteome under acute and chronic heat stress are still lacking in systematic description. In addition, existing research often focuses on a few commonly used varieties, paying insufficient attention to the broader germplasm resources. In reality, the field environment is complex, and various stresses often coexist simultaneously, which also causes a certain gap in the application of laboratory results.

In the future, if this knowledge is to be truly applied to breeding, higher-resolution research is necessary, especially single-cell and spatial transcriptomics, which can depict the specific responses of anthers in each type of cell at high temperatures. Combining these molecular data with field performance and phenotypic analysis in complex environments can also help screen for more reliable markers and candidate genes. Meanwhile, including more local varieties and wild relatives in the research scope may bring about new heat-tolerant alleles or new physiological mechanisms. Ultimately, if CRISPR, genomic selection and validated molecular markers can be combined, the breeding speed of heat-tolerant rice varieties will be significantly accelerated, and it will also be more conducive to stabilizing food production under the condition of continuous climate warming.

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Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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