NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTMENT OF CHEMISTRY

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BSc in Cell and Molecular Biology

# STUDYING THE IMPACT OF HIGH TEMPERATURE ON RICE REPRODUCTION

MASTER IN MSC BIOTECHNOLOGY

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During my university journey, I never had the opportunity to explore the field of plant biology in detail, which made me think that maybe it wasn't appropriate for me. However, after this internship, I realized that I couldn't have been more wrong. There is nothing more satisfying than seeing our work of months develop and result in new plants. During this time, I became more patient and resilient, since working with plants has its challenges, especially the time it takes to get results. Therefore, I would like to thank everyone who shared this one-year adventure with me and helped me discover this new world, plant biology, and its extreme importance.

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# ABSTRACT

Rice (*Oryza sativa L.*) is a staple food for over two-thirds of the global population, yet its productivity is threatened by global warming. Rising temperatures, especially during anthesis, compromise fertilisation and yield, with an estimated 3% rice grain yield loss per 1ºC increase. Developing heat-tolerant rice varieties is therefore a priority. Publicly available transcriptomic data for the heat-tolerant variety Nagina 22 (N22) enabled identification of genes differentially expressed in pistils during anthesis under heat stress (HS). Selected genes were validated through controlled HS assays and gene expression analysis by RT-qPCR. *OsWRKY55* and *OsPLDalpha4* were selected for knockout due to their lower expression level in N22, as compared with Nipponbare, while *OsWRKY53*, a homolog of *OsWRKY55*, was included to account for potential functional redundancy. Using CRISPR-Cas9, rice plants with single-knockouts of *OsWRKY55* and *OsPLDalpha4*, and a double-knockout of *OsWRKY53* and *OsWRKY55* were successfully generated, all producing truncated proteins with loss of function. *OsYABBY7* and *OsLEA17* were selected as upregulated genes in pistils under HS. To further study the function of all selected genes, their expression profile was assessed in pistil of different rice varieties, including varieties grown in Portugal, in response to HS at anthesis. Ariete showed expression patterns potentially associated with higher heat tolerance, while Kitaake demonstrated potentially greater heat-sensitivity. Pistil morphology analysis showed that Ariete, OP2115, and Teti, may have advantages under HS conditions. Grain yield assay under HS was limited by the severity of the heat treatment, since most rice varieties did not produce seeds. Nevertheless, it confirmed that N22 is the most HS-tolerant variety, since it was the only one producing seeds. Altogether, this work allowed us to identify and characterise new genetic targets and to produce new rice genotypes with potentially higher HS tolerance, providing both fundamental knowledge and applied strategies to mitigate the HS effects.

**Keywords:** Rice, Heat Stress, Anthesis, Pistil, Gene Expression, CRISPR-Cas9

# RESUMO

O arroz (*Oryza sativa L.*) é um alimento básico para mais de dois terços da população mundial, mas a sua produtividade é ameaçada pelo aquecimento global. O aumento das temperaturas, especialmente durante a antese, compromete a fertilização e o rendimento, com perda estimada de 3% por cada aumento de 1ºC. Portanto, o desenvolvimento de variedades tolerantes ao calor é uma prioridade. Dados transcriptómicos públicos da variedade tolerante ao calor Nagina 22 (N22) permitiram identificar genes diferencialmente expressos nos pistilos durante a antese, sob stress térmico (ST). Estes foram validados através de ensaios de ST e análise da expressão genética por RT-qPCR. Os genes *OsWRKY55* e *OsPLDalpha4* foram selecionados para *knockout* devido à sua baixa expressão em N22, comparativamente com Nipponbare, enquanto *OsWRKY53*, homólogo de *OsWRKY55*, foi incluído pela possível redundância funcional. Utilizando o CRISPR-Cas9, geraram-se *knockouts* simples de *OsWRKY55* e *OsPLDalpha4* e um *knockout* duplo de *OsWRKY53* e *OsWRKY55*, todos produzindo proteínas truncadas com perda de função. *OsYABBY7* e *OsLEA17* foram selecionados como genes induzidos nas mesmas condições. Para caracterizar a função destes genes, os perfis de expressão foram avaliados no pistilo de diferentes variedades, incluindo variedades cultivadas em Portugal, em resposta ao ST na antese. Ariete apresentou padrões de expressão associados a maior tolerância, enquanto Kitaake tinha um perfil de sensibilidade. A morfologia do pistilo mostrou que Ariete, OP2115 e Teti podem ter vantagens em condições de ST. O ensaio de rendimento sob ST foi limitado pela severidade do tratamento, já que a maioria das variedades não produziu sementes. Contudo, confirmou que N22 é mais tolerante, sendo a única a produzir sementes. No conjunto, este trabalho permitiu identificar e caracterizar novos alvos genéticos e gerar novos genótipos de arroz que poderão ter uma maior tolerância ao calor, fornecendo conhecimentos fundamentais e estratégias aplicadas para mitigar os efeitos do ST.

**Palavras-chave:** Arroz, Stress Térmico, Antese, Pistilo, Expressão Genética, CRISPR-Cas9

# DECLARATION OF AUTHENTICITY

In this work, generative artificial intelligence tools were used, namely ChatGPT-5 (https://chatgpt.com), for grammatical review, text reformulation, and to find specific scientific articles. These tools were employed under the author's supervision, and all generated content was verified for accuracy. The authorship and validation of the content remain entirely the responsibility of the author, and the use of artificial intelligence complies with institutional standards of academic integrity.

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# List of Abbreviations

| | |
|---|---|
| **ABA** | Abscisic acid |
| **bp** | Base pair |
| **CCML** | Co-Cultivation Medium Liquid |
| **CCMS** | Co-Cultivation Medium Solid |
| **cDNA** | Complementary deoxyribonucleic acid |
| **CDPK** | Calcium-dependent protein kinases |
| **CIM** | *Callus*-Inducing Medium |
| **cm** | Centimetre |
| **$CO_2$** | Carbon Dioxide |
| **CPBTs** | Conventional Plant Breeding Techniques |
| **CRISPR-Cas9** | Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 |
| **Ct** | Cycle threshold |
| **dd$H_2O$** | Double distilled water |
| **DNA** | Deoxyribonucleic acid |
| **dT** | Deoxythymidine |
| ***E. coli*** | *Escherichia coli* |
| **EDTA** | Ethylenediamine tetraacetic acid |
| **EIM** | Embryogenic Improvement Medium |

| | |
|---|---|
| **g** | Gram |
| **GMOs** | Genetically Modified Organisms |
| **h** | Hour |
| **ha** | Hectare |
| **HS** | Heat Stress |
| **HSFs** | Heat Shock Factors |
| **HSPs** | Heat Shock Proteins |
| ***hptII*** | *hygromycin phosphotransferase* II (Hygromycin resistance gene) |
| **hyg** | Hygromycin |
| **IAA** | Indole-3-Acetic Acid |
| **InDels** | Insertions and Deletions |
| **Kg** | Kilogram |
| **KO** | Knockout |
| **L** | Liter |
| **LB** | Luria-Bertani |
| **LEA** | Late Embryogenesis Abundant |
| **MAPK** | Mitogen-Activated Protein Kinases |
| **MAS** | Marker-Assisted Selection |
| **mg** | Milligram |
| **min** | Minute |
| **mL** | Millilitre |
| **mM** | Millimolar |
| **MSc** | Master of Science |
| **N22** | Nagina 22 |
| **Nipp** | Nipponbare |

| | |
|---|---|
| **NPBTs** | New Plant Breeding Technologies |
| **$OD_{600}$** | Optic density at 600 nm |
| **Os** | *Oryza sativa* |
| **PA** | Phosphatidic Acid |
| **PCR** | Polymerase Chain Reaction |
| **PDM** | Plantlet Development Medium |
| **PSII** | Photosystem II |
| **QTLs** | Quantitative Trait Locus |
| **RAP-DB** | Rice Annotation Project Database |
| **Rca** | Rubisco activase |
| **RM** | Regeneration Medium |
| **RNA-seq** | RNA sequencing |
| **ROS** | Reactive Oxygen Species |
| **rpm** | Revolutions per minute |
| **RT** | Room Temperature |
| **RT-qPCR** | Reverse Transcription-quantitative PCR |
| **s** | Second |
| **SD** | Standard Deviation |
| **SEM** | Standard Error of the Mean |
| **SgRNA** | Single-guide ribonucleic acid |
| **SM** | Selection Medium |
| **WT** | Wild-type |
| **U** | Unit |
| **µg** | Microgram |
| **µL** | Microliter |
| **µM** | Micromolar |

| | |
|---|---|
| ℃ | Degrees Celsius |

# 1

# INTRODUCTION

## 1.1 Importance of rice and population growth

*Oryza sativa* L., commonly known as Asian cultivated rice, is a staple food for more than two-thirds of the world's population [1]. It is the third most cultivated and consumed crop globally and plays a crucial role in ensuring food security [2]. The importance of rice is particularly pronounced in Asian countries, as they produce 90% of the global supply [3], and provide up to 76% of the caloric intake for the Asian population [4], [5]. However, its relevance is not limited to this continent, as it is also highly important in Europe. Portugal is one of the most outstanding countries on this continent, being the fourth largest rice producer [1]. Annually, Portugal produces approximately 180,103 tons of rice on 30,000 ha, representing 6% of European production and 60% of domestic consumption [6]. In addition, rice consumption in Portugal is the highest in Europe, at 16 kg per capita per year, about three times the European average [7]. Some of the most widely produced varieties in the country are Carolino (*japonica*), such as Ariete, Ceres, Teti, and Agulha (*indica*) types [1]. Notably, over the past 15 years, INIAV, in collaboration with COTArroz, developed the first fully Portuguese variety, Caravela [8]. The Portuguese rice breeding program continues to test and evaluate new varieties, such as OP2115.

Although global rice production is already high, 755 million tons harvested on approximately 164 million hectares, the production needs for the coming years continue to rise [2], [6]. This increasing demand is primarily driven by global population growth. It is estimated that by 2050 a 70% increase in food production will be necessary [5]. The current global population is around 8 billion people and, according to the United Nations, it is predicted that by 2080 the number will reach 10.3 billion [9]. This rapid growth exacerbates production and

consumption inequalities, leading to the aggravation of global warming. Therefore, it is essential to find solutions to improve rice productivity, otherwise there will be a serious food security problem, leading to hunger and malnutrition for a large percentage of the population [10].

## 1.2 Global warming and heat stress as a constraint to rice productivity

Global warming, driven by increased greenhouse gas emissions [11], corresponds to a gradual rise in the Earth's average temperature, which is expected to exceed 1.5°C before 2027 [12]. The main reasons responsible for the emission of these gases are the increase in the world population and its activities, such as the consumption of fossil fuels, deforestation, environmental degradation, agricultural activities, livestock production, among others. And, as a result, atmospheric $CO_2$ concentrations have increased by nearly 50% in the past six decades, reinforcing the greenhouse effect and accelerating global warming [13].

The consequences of this warming are already evident in the form of climate change, characterised by more frequent and extreme weather events, such as increased heat waves, reduced rainfall, rising sea levels, more droughts, storms, etc. [5]. Beyond the rise in temperatures, precipitation and humidity are also expected to increase by 8% and 3%, respectively, and the average drought index might double. These changes enhance the risk of pest and disease outbreaks, placing agriculture in an increasingly vulnerable position and raising serious concerns about global food security [10].

Among the abiotic stresses triggered by climate change, heat stress (HS) is particularly detrimental to crop productivity. It is characterised by a temperature increase of 10-15ºC beyond the threshold level for a period of time capable of causing irreversible damage to plant growth and development [14]. Its impact depends on several factors such as the intensity, duration, rate of increase and variety's inherent heat tolerance [14]. For instance, HS lasting 3-5 days is considered mild, 5-7 days moderate, and over 8 days severe [15]. *Indica-japonica* hybrid varieties generally display the highest heat tolerance, followed by *indica* and *japonica* [15].

The effects of HS on rice are well studied, with yield reductions of approximately 3-10% for every 1°C increase in the global mean temperature [5], [10], [16]. However, the change in yield may depend on the time of day at which the stress occurs, in other words, whether it is during the day or at night. Both types of HS impact yield, fertility, chalkiness, and nutritional quality, with the latter parameter being most affected by nighttime stress [11]. Thus, during

daytime HS, a 6% reduction in yield can be expected for each 1°C increase in the optimal daytime temperature of 28°C, and a 7% reduction for each 1°C increase in the minimum nighttime temperature of 22°C [11]. Additionally, studies show that nighttime temperatures are rising faster than daytime temperatures and, furthermore, nighttime HS tends to persist longer and affects more areas of the world compared to daytime HS [11]. Thus, it represents an invisible natural disaster [17].

In Portugal, climate change will also result in higher temperatures, leading to an increase in the number of hot days and a reduction for cold days. These changes will directly affect rice cultivation, particularly varieties already adapted to local conditions, and may further compromise productivity [1].

## 1.3 Mechanisms involved in plant response to heat stress

### 1.3.1 The impact of heat stress on each stage of rice growth

Rice development is divided into three major phases: vegetative, reproductive, and the grain filling/maturation phases, each one characterised by distinct developmental processes [18]. The vegetative growth phase includes seed germination, seedling emergence, and tillering. This stage is fundamental, as a greater number of tillers directly translates into increased panicle production in later developmental stages. The reproductive phase follows, including panicle initiation and differentiation, flag leaf booting, heading (panicle exsertion), gametophyte development, anthesis (flowering), pollination, and fertilisation. Finally, grain filling and maturation occur. This happens after the fertilisation of the ovary and comprises the stages of milk stage, soft dough stage, hard dough stage, grain-drying stage, and finally, maturity. During these stages, starch and sugars are translocated, accumulated and progressively solidified within grains. At this stage, the process of photosynthesis is particularly important due to the production of starch and sugars [18]. HS negatively compromises all stages of growth in different ways, but leads to the same final result, reduced yield, as seen in **Figure 1.1**.

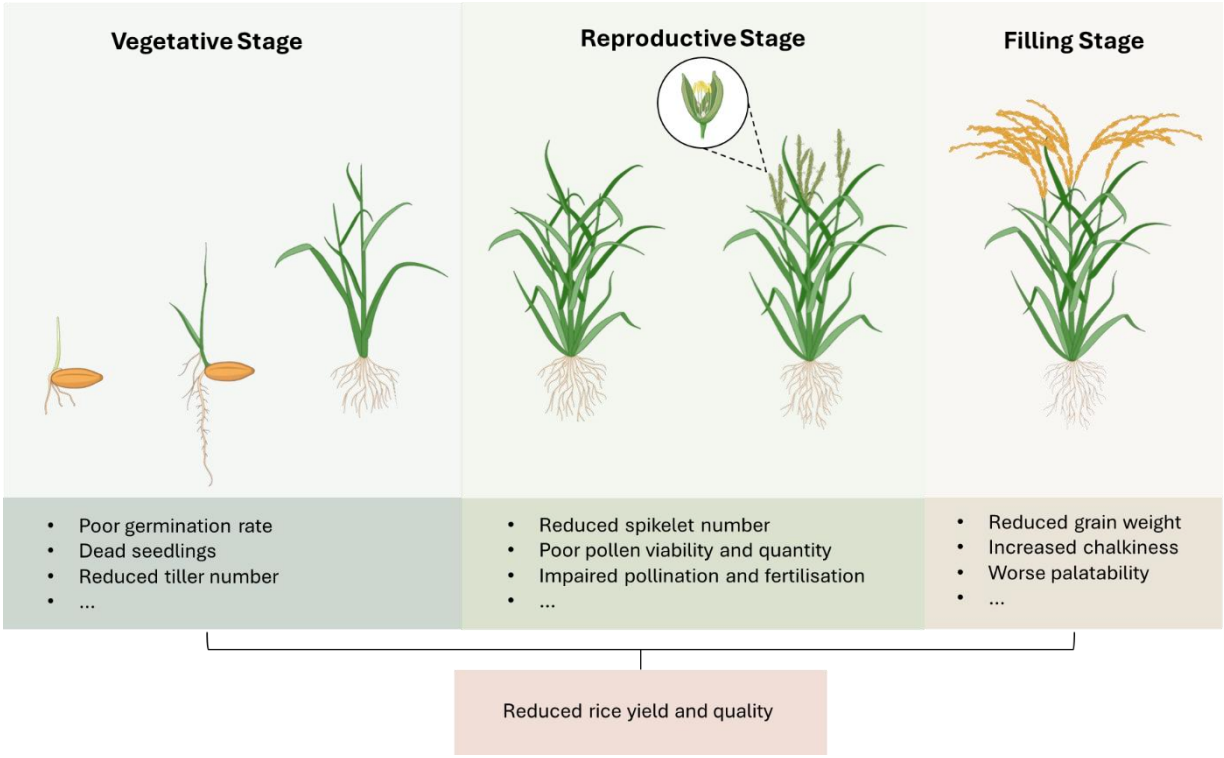


**Figure 1.1 - Morphological and physiological traits triggered by heat stress on each stage of rice growth.**
Adapted from [5].

In the case of vegetative growth, if HS occurs in the early stages, it can lead to reduced seed germination potential, poor germination rate, seedling vigour, or even seedling death. If it occurs in the tillering phase, changes in leaf morphology and a reduction in the number of tillers and biomass are observed, with a possible reduction of 35% in the number of panicles and 86% in yield (40 °C day/35 °C night) [5], [19]–[21].

The reproductive phase is the most susceptible to HS, since all processes that occur during this phase are strongly affected by heat [22]. This is most harmful in the days before flowering, but reaches its peak on the day of anthesis [23]. If it occurs at the initiation and differentiation of the panicle, there may be a reduction in the number and size of spikelets [23], leading to a 66% reduction in yield (40°C day/35°C night at the pre-flowering stage for 15 days) [24]. High temperatures during anther development can cause premature degradation and disintegration of the tapetal cells, defective pollen wall formation, and pollen-grain abortion [20]. The most critical point is during the early microspore stage, after meiosis, leading to a total loss of spikelet fertility. During anthesis, HS impairs pollination and the fertilisation process, leading to a loss of spikelet fertility. This occurs because of anther dehiscence, reduction in pollen number and viability, reduced pollen swelling, poor germination of pollen grains on the stigma, inhibited elongation of pollen tubes, and reduced stigma length [19], [22], [25].

Finally, if it occurs after fertilisation, abnormal cellularization of early endosperm development occurs, impairing the subsequent establishment of endosperm [5]. Collectively, HS at the reproductive stage can lead to a reduction of more than 80% in spikelet fertility [21].

HS during the grain-filling stage results in lower quality rice, reduced palatability, altered appearance and chalkiness, and a reduction in filling time, affecting the starch and other sugar content [19], [26]. HS during the night has a major impact at this stage, as it increases respiration, leading to increased carbohydrate consumption by the plant, which otherwise were destined for filling [5], [18], [19]. Additionally, enzymes involved in starch biosynthesis are sensitive to HS, while those associated with starch degradation are upregulated [22].

In summary, while all developmental stages are vulnerable to HS, the reproductive phase is the most critical for grain yield. Therefore, identifying and characterising tolerance mechanisms at this phase is a priority for developing heat-tolerant rice varieties.

### 1.3.2 Stamen and pistil morphology may attenuate the effects of heat stress

In addition to studying gene expression in reproductive organs, morphological traits of stamens and pistils are also critical for understanding which characteristics may lead to higher natural tolerance in varieties. The stamen consists of six anthers, responsible for pollen production, and the respective filaments that transmit water and nutrients to the anthers and help disperse pollen. The pistil comprises two stigmas that capture pollen, two styles that help pollen reach the ovary, and the ovary itself [27], [28]. These reproductive organs can be seen in **Figure 1.2**.

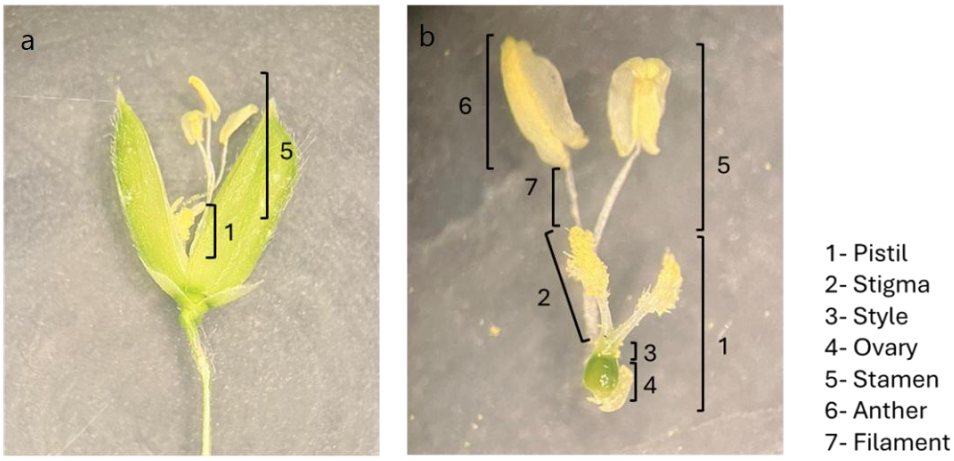


**Figure 1.2 - Morphological structure of a rice spikelet and reproductive organs.** (a) External view of the intact spikelet. (b) Dissected spikelet showing the reproductive organs. Images were obtained from spikelets of Kitaake variety grown under optimal conditions.

Each variety of rice may present morphological variation in these organs, which can benefit the plant by guaranteeing heat tolerance. In the case of the stamen, studies have shown that some positive characteristics are the reduction of the cell layer between the anther locule (where pollen develops) and the lacuna (dehiscence area that connects to the pores), as this makes anther dehiscence more effective and facilitates pollen release [29]. Furthermore, longer anthers, together with larger pores and apical zone, have been associated with more efficient pollen dispersal [29], [30].

There are fewer studies available regarding the pistil. However, one of the most important characteristics involved in HS tolerance is stigma exsertion. This trait refers to the phenomenon where the stigma exits the lemma and palea after the spikelet opens. This allows an increase in the number of pollen grains on the stigma surface, as the area increases and allows the flowering period to be prolonged. Nevertheless, studies have shown that this trait is detrimental at high daytime temperatures, since during this time of day exserted stigmas are directly exposed to intense light and high temperatures, leading to desiccation, oxidative damage, and reduced stigma receptivity [31]. These conditions also cause stamen-pistil desynchrony [17], resulting in poor fertilisation rates [17]. In contrast, during nighttime HS, the absence of solar radiation and higher humidity allow exserted stigmas to retain hydration and receptivity longer, extending the time for pollen capture and fertilisation [17], [31]. Thus, the combination of heat, light, and humidity determines whether stigma exsertion acts as a beneficial or detrimental trait under HS. This characteristic is positively related to stigma and style length [32], [33].

Understanding these morphological traits can guide breeding approaches, allowing the selection of varieties with optimised reproductive structures that enhance fertilisation efficiency under HS.

### 1.3.3 Sensing and molecular responses to heat stress

Plants, as sessile organisms, unlike other living beings, cannot escape unfavourable environmental conditions and abiotic stresses. For this reason, there was a need to evolve in order to develop cellular, morphological, and physiological adaptation mechanisms, as shown in **Figure 1.3**. When these mechanisms fail, irreversible damage may occur at the cellular level, compromising the viability of the cells and the plant [13].

The HS response process is initiated by the sensing of stress signals such as changes in ions, temperature alterations, and changes in membrane fluidity. The cell membrane is the first

structure affected, as high temperatures reduce its fluidity [19], potentially altering the function and structure of membrane-associated proteins, which act as primary sensors [20]. Following these changes, a flow of $Ca^{2+}$ ions is generated, leading to increased expression of kinases such as mitogen-activated protein kinases (MAPK) and calcium-dependent protein kinases (CDPK). After the signalling cascade is activated, significant alterations begin to occur in the cells, in order to mitigate heat-induced damage. Reactive oxygen species (ROS), produced under HS, are scavenged by the enhanced production of antioxidants to prevent severe oxidative damage to the cells [19]. Simultaneously, due to water loss, osmolytes are produced to restore osmotic balance. At the transcriptional level, gene expression is also significantly modified, favouring the expression of heat shock factors (HSFs) and the synthesis of heat shock proteins (HSPs). These proteins function as molecular chaperones, assisting in protein folding, preventing aggregation, and maintaining protein stability under HS conditions. They also play an important role in signal transduction and in the activation or repression of specific genes that lead to increased tolerance in the plant [23]. In addition, HS can interfere with post-transcriptional modification as mRNA splicing, further influencing gene expression.

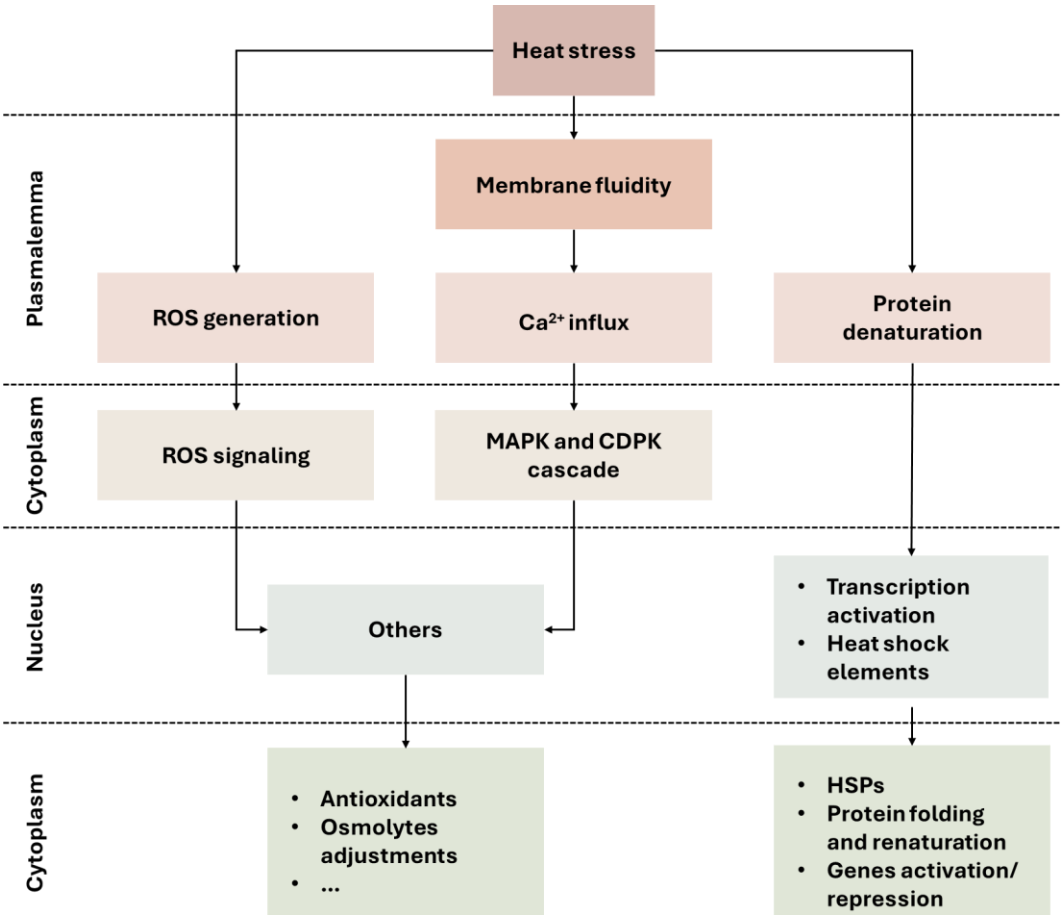


**Figure 1.3 - Scheme representing the sensing and molecular responses of plants under heat stress.** Adapted from [14].

### 1.3.4 Morphological, anatomical, and physiological responses at the cellular level

Beyond cellular and molecular signalling, HS causes various morphological, anatomical, and physiological responses that affect overall plant performance. Morphologically, general alterations can be observed, such as burns on leaves and stems, inhibition of leaf and root growth, leaf senescence and abscission, and ultimately reduced yield [5], [14]. More specific changes are observed, depending on the plant developmental stage, as described above in "The impact of heat stress on each stage of rice growth".

At the anatomical level, HS leads to a reduction in cell size, alterations in xylem vessels, and increased stomatal density coupled with stomatal closure to minimise water loss [14]. Chloroplasts also undergo significant structural modifications, particularly in thylakoid membranes, which impair photosystem II (PSII) activity and reduce photosynthetic efficiency [14].

Physiologically, HS disrupts water balance, accelerating transpiration and reducing root water uptake due to decreased hydraulic conductivity [34]. Plants respond by accumulating osmolytes such as proline, glycine, betaine, and other soluble sugars to maintain osmotic homeostasis and stabilise proteins and membranes [5]. Photosynthesis is also affected, not only by thylakoid and PSII damage, but also due to chlorophyll degradation and the thermosensitivity of rubisco activase (Rca) [23]. Additionally, ROS accumulation can damage the apparatus and inhibit protein synthesis, further decreasing photosynthetic rates and biomass accumulation, with reductions of yield up to 20% reported at 42ºC [5], [19], [23]. Heat also affects membrane fluidity, leading to cellular damage and electrolyte leakage [5]. The hormonal response also changes, specifically abscisic acid (ABA), ethylene, cytokinins, auxins, and gibberellins. In the case of ABA and ethylene, HS benefits the production of both. The first favours an increase in ROS in the anthers, leading to the induction of programmed cell death in the tapetum and pollen sterility [20]. The second is associated with an increase in water-saving capacity [20]. Cytokinins tend to decrease in concentration, leading to a reduction in chlorophyll and a delay in the grain filling stage, which can also decrease spikelet numbers [20]. Auxin reduction disrupts the indole-3-acetic acid (IAA) pathway, leading to spikelet sterility, while gibberellins respond to ROS accumulation by activating starch-degrading enzymes, increasing seed chalkiness [20]. Finally, secondary metabolites such as flavonoids, phenolics, and antioxidants are synthesised to mitigate oxidative damage caused by ROS [23].

## 1.4 Different strategies to mitigate heat stress

With the dual challenges of rapid population growth and global warming, it is essential to implement effective strategies to increase rice productivity. These strategies must be supported by policies and investments at both farmer and research levels [1]. To accomplish these goals, a combination of agronomic, breeding and biotechnological strategies can be used.

Several agronomic practices can contribute to mitigating the effects of HS, including adjusting planting dates to avoid the warmest periods of the year, altering crop rotations, and implementing improved irrigation systems [13], [14], [19]. Another strategy is to develop new varieties that are more tolerant to stress, that is, plants with the ability to grow and maintain their yield at high temperatures [14]. Two different methods can be used: the traditional method, based on conventional plant breeding techniques (CPBTs) between different varieties, and the biotechnological method, using new plant breeding technologies (NPBTs) through genetic engineering.

The traditional approach, CPBT, relies on breeding programs that evaluate and select elite germplasm with desirable agronomic traits, such as being capable of achieving good yields and fertility under HS conditions. Screening for tolerance is often carried out under controlled conditions, in growth chambers or glasshouses, to minimise the influence of other stress factors during the evaluation. However, not all laboratories are able to have these facilities, making it difficult to properly evaluate the tolerance of varieties [14]. In contrast, NPBTs have been gaining popularity because they are more efficient, robust, cheaper, and less time-consuming when compared to CPBT [10]. This strategy allows an increase in tolerance through deeper molecular and genetic understanding of stress responses, and can be performed through molecular marker-assisted selection (MAS) or genome editing techniques.

Molecular marker technologies, such as high-density quantitative trait locus (QTL) mapping, have been used to identify genes and alleles associated with stress tolerance and to characterise germplasm diversity [19], [23]. MAS technology allows the introgression of heat-tolerant QTLs from tolerant donor plants into elite but sensitive varieties, thereby accelerating breeding progress while retaining desirable agronomic characteristics [19].

Among NPBTs, Clustered Regularly Interspaced Palindromic Repeats-CRISPR-associated Protein (CRISPR-Cas9) technology has emerged as a powerful tool due to its simplicity, precision, and high efficiency [10]. This system can be applied to edit, insert exogenous genes, or silence or overexpress genes that are negative or positive regulatory, respectively [20]. Despite its potential, the use of CRISPR-Cas9 in agriculture faces regulatory and societal challenges,

particularly in European Union, where genetically modified organisms (GMOs) are restricted, in order to protect human and animal health and the environment, and ensure the functioning of the internal market [35]. Nonetheless, by using this technology, it is possible to genetically edit organisms without leaving marks of exogenous DNA, thus facilitating their regulatory acceptance and commercialisation [10].

Furthermore, as relevant tolerance traits have been unintentionally lost over the years during selection processes, it may be interesting to analyse wild relatives of rice. Through them, various heat-tolerant QTLs and genes capable of increasing tolerance [20], [33] may be found. In addition, the *indica* variety, Nagina 22 (N22), is also a good source of QTLs, since it is commonly used as a target for study and breeding, as it has good heat tolerance but poor agronomic performance [25].

Taken together, overcoming the negative impacts of HS may require an integrated strategy that combines conventional breeding with modern genome editing technologies, always supported by sustainable agronomic practices.

## 1.5 Context and rationale of this study

Gonzalez-Schain *et al.* (2016) reported the gene expression profile of the tolerant variety, N22, under optimal and HS conditions, during anthesis, using RNA sequencing (RNA-seq) [25]. This study identified several families of transcription factors, signal transduction genes, and metabolic pathways that are downregulated under stress conditions. In contrast, upregulated genes encoding heat shock factors and other proteins were also discovered. These findings identified different genes as potential candidates for increasing the heat tolerance of rice varieties through biotechnological genome editing approaches. Complementary genomic information available through the Rice Annotation Project Database (RAP-DB), which provides gene sequences, transcript variants, and their expression throughout the stages of growth and in different types of tissue for the Nipponbare (Nipp) variety (*japonica* type) [36], provided additional information that allows identification of the most promising candidates.

In a collaborative work between the Plant Gene Regulation laboratory at ITQB NOVA and the SPReD laboratory at the Faculty of Sciences of the University of Porto, they aimed to identify genes that could be candidates for enhancing the tolerance of the heat-sensitive Nipp rice variety. To this aim, a group of genes differentially expressed during anthesis in the reproductive tissues of N22 under HS was selected based on the study by Gonzalez-Schain *et al.* (2016) [25]. These genes were then filtered using RAP-DB to ensure their expression was specific to

the pistil [37]. Following this gene selection, an assay was conducted in PGR lab at ITQB NOVA using N22 and Nipp plants subjected to either control or HS conditions, during anthesis. After heat treatment, the pistils were sampled, and a gene expression analysis (RT-qPCR) was performed at Porto University to assess differential expression of candidate genes between treatments and varieties.

The gene expression analysis allowed the selection of genes that were good candidates for knockout to increase the tolerance of the sensitive variety Nipp. These genes are downregulated in N22 as compared with Nipp, under control conditions, and/or are downregulated in Nipp under HS, but not in N22. Thus, genes *OsPLDalpha4* and *OsWRKY55* were selected (**Figure 1.4**). Regarding *OsPLDalpha4*, it is clear that its expression remains reduced in both control and HS situations in N22 as compared to Nipp, and Nipp shows a significant downregulation under HS. Even so, the expression in Nipp under HS remains higher than that of N22, with possibilities for further downregulation. In the case of *OsWRKY55*, a different situation was observed. Its transcript level is lower in N22 as compared to Nipp and in N22 a downregulation is observed under HS. In Nipp, however, expression remains high, with no changes between control and HS. Thus, as with *OsPLDalpha4*, the knockout of this gene might be advantageous in a HS-sensitive variety such as Nipp.

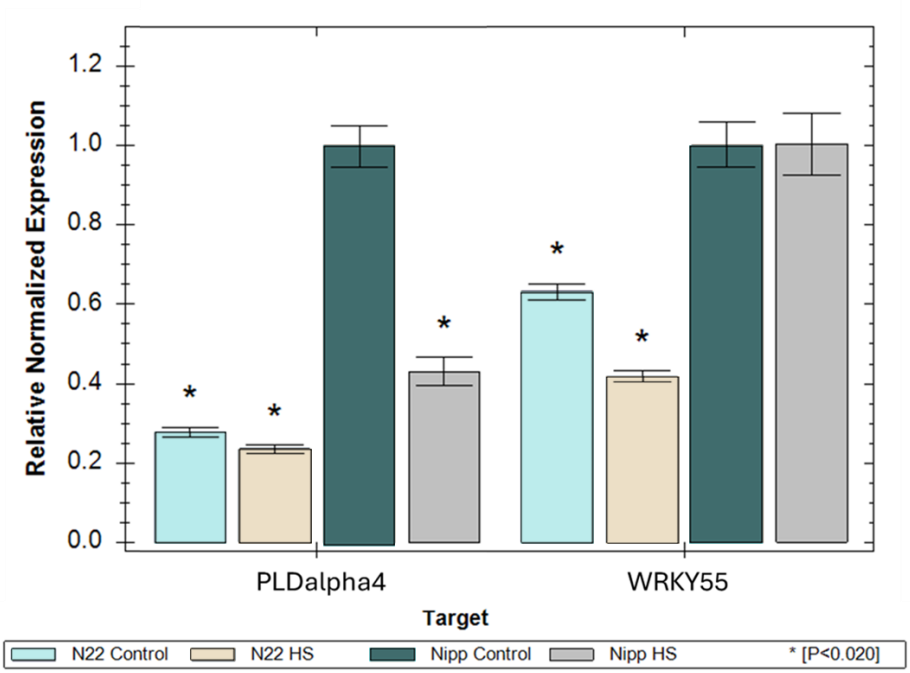


**Figure 1.4 - Gene expression analysis of *OsPLDalpha4* and *OsWRKY55* genes in the pistils of Nagina 22 and Nipponbare varieties pollinated under heat stress.** RT-qPCR data obtained in SPReD laboratory at the Faculty of Sciences of the University of Porto.

As the *OsWRKY53* gene is homologous to *OsWRKY55* within the WRKY domain [38], it may be involved in the same pathways associated with the HS response. Therefore, in addition to performing single-knockouts of the *OsWRKY55* and *OsPLDalpha4* genes in Nipp, it was decided to perform a double-knockout for the *OsWRKY53* and *OsWRKY55* genes.

In parallel, a HS treatment was conducted on rice varieties grown in Portugal, namely Ariete, Caravela, Ceres, OP2115, and Teti, and on control varieties, Kitaake, N22, and Nipp. This assay aimed to assess, in these varieties, the expression of the genes under study (HS-responsive genes) in response to HS, as well as the effect on the agronomical parameters. Gene expression analysis was focused on *OsWRKY55, OsPLDalpha4, OsWRKY53, OsYABBY7*, and *OsLEA17*, the last two selected due to their reported upregulation under HS and pistil-specific expression. The main goal was to identify rice varieties exhibiting gene expression patterns similar to the heat-tolerant variety N22. Additionally, during pistil isolation, it was decided to analyse their morphology, trying to associate it with their level of HS tolerance. Finally, to try to corroborate the theories based on the two previous topics, gene expression and pistil morphology, and understand the tolerance of the varieties, an agronomical parameters analysis for these varieties was performed under HS and control conditions.

### 1.5.1 Information about the genes of interest

The genes selected to study their expression and influence on HS, *OsWRKY55, OsPLDalpha4, OsWRKY53, OsYABBY7*, and *OsLEA17*, are quite distinct from each other, belonging to different gene families and, consequently, presenting different functions and modes of action. *OsWRKY53* and *OsWRKY55* belong to the WRKY transcription factor family, characterised by the presence of a WRKY-DNA-binding domain [39]. This family is commonly related to a wide range of abiotic stresses and frequently operate through MAPK signalling pathways, as observed in these two genes [39], [40]. Regarding *OsWRKY53*, there are studies that demonstrate how its expression negatively affects cold tolerance during the reproductive phase, more specifically, the booting stage. This happens because there is a repression of GA biosynthesis in the anthers, affecting the correct development of pollen [41]. As for *OsWRKY55*, it has been shown to be induced by drought. However, its over-expression leads to increased water loss, enhanced accumulation of ROS, and consequently lower tolerance. In addition, there is a reduction in plant size due to decreased cell elongation [39]. Therefore, knocking out these two genes could enhance rice tolerance to cold and drought. Moreover, since drought is often

present during HS, reducing the expression of these genes may provide valuable insights into their potential role in multi-stress tolerance.

The *OsPLDalpha4* gene belongs to the phospholipase enzyme family, which principal function is to modify lipids to generate phosphatidic acid (PA), a second messenger involved in abiotic stress signalling and membrane remodelling. Members of this family contain a C2 domain, which mediates calcium-dependent membrane binding and two conserved HKD motifs related to catalysis. They are involved in abiotic stress signalling, membrane remodelling, defence, hormonal signalling, among others [42], [43]. *OsYABBY7* belongs to the YABBY transcription factors family, defined by a C2C2 zinc-finger domain at the N-terminus, and a YABBY DNA-binding domain at the C-terminus. They regulate reproductive development, affecting meristem maintenance, spikelet structure, and leaf development [44]. Finally, *OsLEA17* belongs to the Late Embryogenesis Abundant (LEA) gene family and the translated proteins are highly hydrophilic and disordered. They are known for being responsible for increasing some stress response proteins synthesis and, therefore, are normally found in an upregulation situation [45]. It is important to note that there are no studies demonstrating the impact of these specific genes on HS, or for other abiotic stresses, such as drought, salinity, cold, etc.

## 1.6 Main goals

Given the global importance of rice, the increasing demand for food production, and the negative impact of HS on yield, it is critical to understand the molecular mechanisms underlying HS responses in order to develop more tolerant rice varieties. The main objective of this study is to better understand the molecular mechanisms underlying the HS response at anthesis and to generate rice varieties with enhanced tolerance to HS through target knockouts of *OsWRKY55*, *OsPLDalpha4*, and *OsWRKY53/OsWRKY55* genes. Furthermore, from a national perspective, this study also aims to characterise the expression pattern of the genes of interest (*OsWRKY55*, *OsPLDalpha4*, *OsWRKY53*, *OsYABBY7*, *OsLEA17*) across rice varieties cultivated in Portugal (Ariete, Caravela, Ceres, OP2115, and Teti), as well as in model varieties (Kitaake, N22, Nipp). The transcriptional profile of the different rice varieties may provide insights into their relative heat tolerance. Additionally, given the limited knowledge regarding the influence of pistil morphology on HS tolerance, this study aims to evaluate pistil structural traits and correlate them with potential tolerance. Finally, to validate hypotheses derived from gene expression and pistil morphological analysis, the study will assess the yield (agronomical parameters) of these varieties under optimal and HS conditions during anthesis. This will allow the integration

of molecular, morphological, and agronomical data to identify rice germplasm with enhanced HS tolerance.

## 1.7 Thesis outline

The main objective of the thesis is to investigate the molecular mechanisms of the response and the impact of high temperatures on rice (*Oryza sativa L.*) reproduction, particularly during anthesis, the most heat-sensitive developmental stage.

The introduction presents the global and national importance of rice as a staple food and highlights the challenges associated with global warming to food security. It discusses how rising temperatures induce HS that compromises fertilisation and yield. The physiological, molecular, and morphological responses of rice to HS are reviewed, along with mitigation strategies, including breeding and biotechnological approaches. Finally, the context and specific objectives of this study are presented.

The materials and methods describe the processes for generating CRISPR-Cas9-edited plants (vector construction for CRISPR-Cas9, *Agrobacterium* transformation, rice transformation, and plant genotyping), to perform morphological and gene expression analysis of different varieties under optimal and HS conditions (plant growth, isolation and observation of pistils, RNA extraction and purification, cDNA synthesis, and optimisation of primers for qPCR), and to perform a yield assay (design of experiment and evaluation of yield).

The results and discussion integrate findings from mutant generation, morphological and gene expression analysis, and yield assessment. The efficiency of the mutant generation was evaluated, and the responses of different rice varieties under control and HS were analysed. These results are discussed using literature to elucidate potential mechanisms of HS tolerance and the functional role of the selected genes.

Finally, the conclusions and future perspectives summarise the main findings and proposing new experiments to complement the data obtained in this study.

# 2

# MATERIALS AND METHODS

## 2.1 Generation of CRISPR-Cas9 rice edited plants

This section will describe the different steps involved in the production of transgenic rice plants with a CRISPR-Cas9 construction to edit the genes of interest: generation of CRISPR-Cas9 vector constructs, Agrobacterium transformation, and rice transformation. The last two steps are summarised in **Figure 2.1**.

### 2.1.1 CRISPR-Cas9 vector constructs

To obtain the CRISPR-Cas9 vector constructs required to knockout the genes of interest in rice, advanced cloning technologies were used. Three different final constructs were generated, two containing a single gRNA for the *OsWRKY55* or *OsPLDalpha4* genes (previously generated), and another with the gRNAs for *OsWRKY53* and *OsWRKY55* genes. The first two were obtained previously, by another student, while the third one was generated during this study using Golden Gate technology.

#### 2.1.1.1 Vectors used to obtain the CRISPR-Cas9 construct to edit both *OsWRKY53* and *OsWRKY55* genes

To assemble the CRISPR-Cas9 vector construct carrying a double gRNA, five vectors were used. L1P1 (Addgene Plasmid #68263) has the hygromycin (hyg) resistance cassette, which serves as a selection marker for future rice transformations. The L1P2 (Addgene Plasmid #68258) carries the Cas9 expression cassette, essential for inducing the double-strand breaks in the target genes. The L1P3 (Addgene Plasmid #112027) and L1P4 (Addgene Plasmid #112028), both driven by the U6 promoter, have the single-guide ribonucleic acid (sgRNA)

cassette for the *OsWRKY55* and *OsWRKY53* genes, respectively. L1P3 includes recognition sites for the type IIS *Bsa*I endonuclease, while the second contains sites for *Esp*3I.

Initially, digestion-ligation reactions were performed to insert the corresponding sgRNAs into L1P3 and L1P4. Subsequently, a final assembly reaction was carried out to combine all level 1 vectors (L1P1, L1P2, L1P3, and L1P4) into the level 2 destination vector, L2 (Addgene Plasmid #48037). All level 1 vectors contain an ampicillin resistance gene, while the L2 vector carries a spectinomycin resistance gene for bacterial selection.

#### 2.1.1.2 SgRNA design and annealing reaction

To begin the process, the forward and reverse oligonucleotides to make the sgRNAs for both *OsWRKY53* and *OsWRKY55* genes were designed. To facilitate the cloning of the two sgRNAs into the entry vectors, specific adapters were added to the oligonucleotides (**Table 2.1**). These adapters ensured correct alignment with the U6 promoter sequences in each vector: for L1P3, the terminal G of the promoter was included in the adaptor, and for L1P4, additional nucleotides were incorporated to restore the missing regions of the promoter.

**Table 2.1 - Oligonucleotides designed for *OsWRKY53* and *OsWRKY55* genes.** The adapters are indicated by the colour red.

| Primer | Sequence | Length (bp) | Tm (°C) | %GC |
|---|---|---|---|---|
| *OsWRKY53*_U6_FW | CAGACTTGTGAACGACGTGATGAACGGGTTT | 31 | 72.7 | 48.4 |
| *OsWRKY53*_U6_RV | TCTAAAACCCGTTCATCACGTCGTTCACAAG | 31 | 71.0 | 45.2 |
| *OsWRKY55*_U6_FW | CTTGATTGATGCGGACTCGGCAC | 23 | 68.8 | 56.5 |
| *OsWRKY55*_U6_RV | AAACGTGCCGAGTCCGCATCAAT | 23 | 70.2 | 52.2 |

To assemble the *OsWRKY53* and *OsWRKY55* sgRNAs, annealing reactions were carried out using the corresponding forward and reverse oligonucleotides for each gene of interest. Each 50 µL reaction contained 2 µL of each oligo (100 µM) and the remaining volume of annealing buffer (1X), and was performed according to the program shown in **Table 2.2** in a UNO II thermocycler (Biometra, Germany).

Table 2.2 - Annealing reaction program.

| Reaction Step | Temperature (ºC) | Time | Cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5′ | 1 |
| Denaturation | 95 | 1′ and decrease 1ºC per cycle until annealing temperature is reached | 1′ x 35 cycles |
| Annealing | 60 | 30′<br>1′ and decrease 1ºC per cycle until 25ºC | 1′ x 35 cycles |
| Pause | 8 | ∞ | |

#### 2.1.1.3 Digestion and ligation of sgRNAs into L1P3 and L1P4 vectors

To insert the sgRNAs into their respective vectors, *OsWRKY55* sgRNA into L1P3 and *OsWRKY53* sgRNA into L1P4, digestion-ligation reactions were performed. For this purpose, two separate 20 µL reactions were prepared, each containing 1 µL of the corresponding vector (L1P3 or L1P4, 100 ng), 1 µL of the annealed sgRNA (*OsWRKY55* or *OsWRKY53*), 1 µL of T4 DNA reaction Buffer (10X), 0.5 µL of T4 Ligase (5 U/µL), 0.5 µL of the appropriate restriction enzyme (*Bsa*I or *Esp*3I), and nuclease-free water. The program utilized is shown in **Table 2.3** and was performed in a thermocycler.

Table 2.3 - Digestion-ligation reaction program.

| Reaction Step | Temperature (ºC) | Time | Cycles |
|---|---|---|---|
| Step 1 | 37 | 20′′ | 1 |
| Step 2 | 37 | 3′ | x 26 cycles |
| Step 3 | 16 | 4′ | |
| Step 4 | 50 | 5′ | |
| Step 5 | 80 | 5′ | 1 |
| Pause | 4 | ∞ | |

To select the vectors properly constructed, 1 µL of either L1P3_*WRKY55* or L1P4_*WRKY53* digestion-ligation was heat shock transformed into 100 µL of *Escherichia coli* (*E. coli*) strain DH5α competent cells [46], which were then plated on LB plates containing 100 mg/L

ampicillin, 0.1 mM IPTG, and 200 µg/mL X-gal, and incubated overnight at 37°C. The selected transformed colonies, the white ones, were inoculated and grown into 5 mL of liquid LB medium, containing 100 mg/L ampicillin, at 37ºC overnight. The plasmid DNA from this culture was extracted using the NZYMiniprep kit (NZYtech, Portugal), following the manufacturer's instructions. Finally, Sanger DNA sequencing was performed to confirm the presence and correct insertion of the sgRNA sequences.

#### 2.1.1.4 Assembly of the final Golden Gate level 2 vector

The final reaction allowed the assembly of all four Golden Gate level 1 vectors into the level 2 destination vector. A 10 µL reaction was prepared, containing 1 µL of each level 1 vector (300 ng), 1 µL of the level 2 destination vector (100 ng), 1 µL of T4 DNA reaction buffer (10x), 1 µL T4 Ligase (5 U/µl), 0.5 µL of the restriction enzyme *BpiI*, and nuclease-free water. The program is shown in **Table 2.3**.

Following the assembly, *E. coli* strain DH5α competent cells were heat shock transformed with 1 µL of the resulting level 2 vector (L2_dgRNA_*WRKY55*_*WRKY53*) [46], plated on LB agar with 100 mg/L spectinomycin, 0.1 mM IPTG, and 200 µg/mL X-gal, and incubated overnight at 37°C. The transformed white colonies were selected, and LB liquid cultures were made with 100 mg/L spectinomycin. The plasmid DNA was extracted using the NZYMiniprep kit, according to the manufacturer's instructions. Lastly, to analyse whether the vector obtained was correct, a plasmid DNA restriction was performed with the enzyme *SalI* (10 U/µL) and analysed by 1% gel electrophoresis. The gel was prepared with GreenSafe Premium (NZYTech, Portugal), and 10 µL of each digested plasmid sample along with 7 µL of GeneRuler DNA Ladder Mix (Thermo Fisher Scientific, USA) were loaded. In addition, plasmid DNA samples were sent for Sanger sequencing.

### 2.1.2 Agrobacterium transformation with the CRISPR-Cas9 constructs

Before performing the *Agrobacterium tumefaciens*-mediated rice transformation, it was necessary to transform them with the CRISPR-Cas9 constructs. For this, 1 µL of the desired plasmid DNA was added to a 50 µL aliquot of *Agrobacterium tumefaciens* strain EHA105 without thawing it. Then, the bacterial cells were subjected to a heat shock at 37°C for 6 min, followed by incubation on ice for 30 min. To facilitate the recovery and increase transformation efficiency, SOC medium (4x the volume of the cells) was added, and the suspension was incubated for 1h at 28°C, with agitation (180 rpm). To concentrate the cell liquid culture, this was centrifuged at 4000 rpm for 4 min at room temperature (RT), and the supernatant was removed

to obtain a final volume of 100 µL. A MYB agar plate with rifampicin (25 µg/mL) and spectinomycin (100 µg/mL) was inoculated with the cell suspension and incubated at 28°C for 3 days.

To investigate whether the colonies were transformed, a colony polymerase chain reaction (PCR) was performed targeting the *hygromycin phosphotransferase*-II (*hptII*) gene (hyg resistance marker). Each 10 µL PCR reaction contained 2 µL of enzyme buffer (Promega, USA), 0.8 µL of $MgCl_2$ (Promega, USA), 0.2 µL of dNTPs (10 mM), 0.4 µL of each primer (10 µM) (**Table 2.4**), 0.08 µL GoTaq G2 Flexi DNA polymerase (Promega, USA), nuclease-free water, and a small amount of bacterial colony as DNA template. Water was used as a template negative control. The PCR program is described in **Table 2.5**, and was carried out in a thermocycler. A 1% agarose gel electrophoresis was prepared to run the PCR products and verify the presence of the amplicon and consequent correct transformation of the Agrobacterium colonies.

The positive colonies were plated in new MYB agar plates with rifampicin (25 µg/mL) and spectinomycin (100 µg/mL), and after an incubation at 28°C for 3 days, Agrobacterium was used for rice transformation.

**Table 2.4 - Primers used for colony PCR.**

| Primer | Sequence | Length (bp) | Tm (ºC) | %GC |
|---|---|---|---|---|
| *hptII* Fw | AATAGCTGCGCCGATGGTTTCTACA | 25 | 66.0 | 48.0 |
| *hptII* Rv | AACATCGCCTCGCTCCAGTCAATG | 24 | 67.0 | 54.2 |

**Table 2.5 - PCR program to amplify the *hptII* fragment.**

| Reaction Step | Temperature (ºC) | Time | Cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5′ | 1 |
| Denaturation | 95 | 45′′ | X 35 cycles |
| Annealing | 60 | 45′′ | |
| Extension | 72 | 40′′ | |
| Final extension | 72 | 10′ | 1 |
| Pause | 8 | ∞ | |

## 2.1.3 Agrobacterium-mediated rice transformation

After making the CRISPR-Cas9 constructions and introducing them in Agrobacterium, the next step is to transform rice embryogenic *calli* and regenerate transformed seedlings. To

obtain rice embryogenic *calli*, Nipp rice seeds were dehusked, followed by surface sterilization. Seeds were incubated in a Benlate solution (1 g/L) (Sigma-Aldrich, USA) for 30 min in a 50°C water bath, treated with 70% ethanol for 2 minutes, and subsequently with 50% bleach solution with a drop of 20% Tween® for 15 minutes under constant agitation. Finally, seeds were rinsed 6-8 times with autoclaved double distilled water ($ddH_2O$) and air-dried under sterile conditions. Sterilized seeds were then placed on *callus* induction medium (CIM), and incubated in the dark at 28ºC for 2 to 3 weeks. During this time, the seeds began their dedifferentiation process, developing 3 distinct structures: seed, *scutella*, and radicle. In the next step, the *scutellum* was separated from the remaining structures and transferred to fresh CIM plates, incubated under the same conditions for an additional 14 days. Subsequently, embryogenic *calli*, totipotent structures capable of producing plantlets, were selected and placed on a new CIM plate, in the dark, at 28ºC for 3 more days.

The next step was *calli* transformation with the transformed Agrobacterium. To do this, a solution (1:1000) of Co-Cultivation Medium Liquid (CCML) with acetosyringone (100 µM) was prepared and Agrobacterium culture harbouring the desired construct was suspended until an $OD_{600}$ of 0.65-0.70 was obtained. The embryogenic *calli* were immersed in this suspension and gently stirred for 15 minutes. The bacterial suspension was discarded, the *calli* were air-dried and placed on Co-Cultivation Medium Solid (CCMS) for 3 days at room temperature (RT), in the dark, wrapped in aluminium foil.

To separate the transformed *calli* from the untransformed ones, they were subjected to three cycles in selection medium (SM), incubated at 28ºC in the dark for 11 days per cycle. As this medium contains hyg, only transformed *calli*, which can express the *hptII* gene, can survive and grow. To enhance the growth and embryogenic potential of the transformed *calli*, the *calli* were then transferred to embryogenic improvement medium (EIM) for 14 days at 28ºC in the dark. Subsequently, the *calli* were passed to regeneration medium (RM) for six to eight cycles, each lasting for 12 days at 32°C under continuous light in a growth chamber. Once small plantlets began to develop, they were transferred to plantlet development medium (PDM) at 32ºC under constant light. This medium also contained hyg, allowing the selection of transformed plantlets. The composition of the solutions and the media used are described in **Tables 5.1 and 5.2** [47].

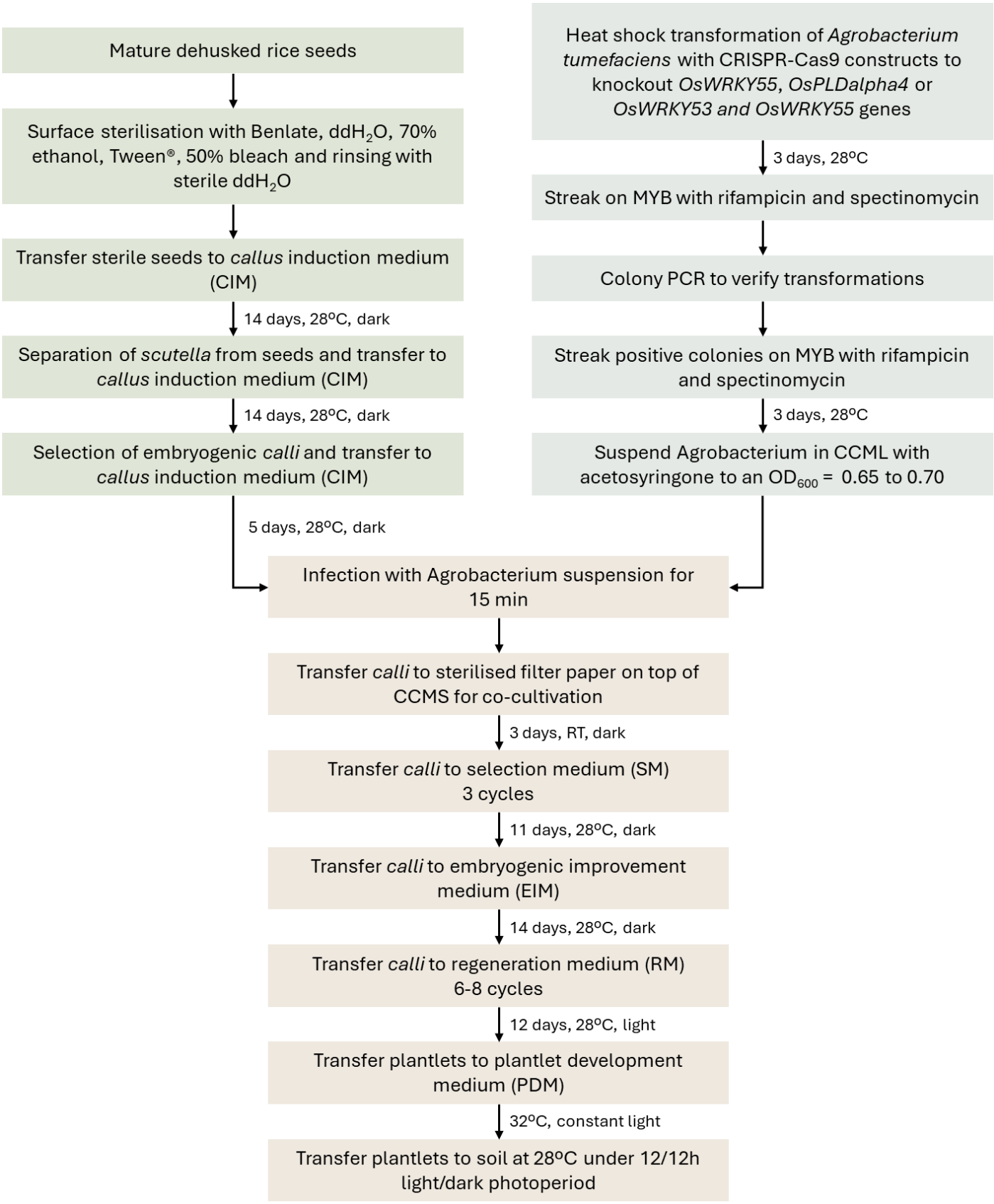


**Figure 2.1 - Summary of the rice transformation protocol.** Adapted from [47].

### 2.1.4 Genotyping rice plantlets regenerated after *calli* transformation

To verify whether the plantlets were transformed, leaf samples were collected, and genomic DNA was extracted using 20 µL of the QuickExtract™ DNA Extraction Solution (Lucigen Cooperation, USA), according to the manufacturer's instructions. Then, a PCR was performed to amplify the *hptII* gene in a 20 µL reaction containing 4 µL of enzyme buffer, 1.6 µL of $MgCl_2$, 0.4 µL of dNTPs (10 mM), 0.8 µL of each primer (10 µM) (**Table 2.4**), 0.16 µL of GoTaq G2 Flexi DNA polymerase, 1 µL of DNA, and nuclease-free water. DNA from a positive plant, previously tested, was included as a positive control, while DNA from a wild-type (WT) plant and water were used as negative controls. The amplification program is described in **Table 2.5**, and it was carried out in a thermocycler. PCR products were analysed in a 1% agarose gel electrophoresis.

For plantlets testing positive for *hptII*, PCR was also carried out to amplify the genes of interest for subsequent Sanger DNA sequencing and analysis of the edits (InDels). Thus, a 50 µL reaction was performed with 10 µL of Taq buffer, 4 µL of $MgCl_2$, 1 µL of dNTPs (10 mM), 2 µL of each primer (10 µM) (**Table 2.6**), 0.4 µL of GoTaq G2 Flexi DNA polymerase, 1 µL of DNA, and nuclease-free water. DNA from a WT plant was used as positive control, and water was used as a negative control. The amplification program is described in **Table 2.7**, and it was performed in a thermocycler. PCR products were also analysed in a 1% agarose gel electrophoresis.

The hyg-positive plantlets were transferred to 2 L pots containing a soil mixture of peat moss, soil and vermiculite (2:2:1 ratio). They were grown in a growth chamber at 28°C/24ºC (light/dark), with a 12h light/12h dark photoperiod, and a light intensity of 500 µmol $m^{-2}$ $s^{-1}$.

**Table 2.6 - Primers used to amplify the edited regions of the rice genes *OsWRKY55*, *OsPLDalpha4*, and *OsWRKY53*.**

| Primer | Sequence | Length (bp) | Tm (ºC) | %GC |
|---|---|---|---|---|
| *OsWRKY55*_FW | GTGCCCTAGTTTGGTACGCT | 20 | 65.2 | 55.0 |
| *OsWRKY55*_RV | CTGCAATGAGACAGGCGAGA | 20 | 65.2 | 55.0 |
| *OsPLDalpha4*_FW | CGAACCTCGCCAATGTTTTTCT | 22 | 64.8 | 45.5 |
| *OsPLDalpha4*_RV | CGTGACGTCGTTCCAGACC | 19 | 65.5 | 63.2 |
| *OsWRKY53*_FW | CTGGCTGAGCTGTGGTACG | 19 | 65.4 | 63.2 |
| *OsWRKY53*_RV | GTACCTCTGAGCCGGGATTG | 20 | 65.0 | 60.0 |

Table 2.7 - PCR program used to amplify the edited regions of the genes *OsWRKY55*, *OsPLDalpha4*, and *OsWRKY53*.

| Reaction Step | Temperature (ºC) | Time | Cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5′ | 1 |
| Denaturation | 95 | 45′′ | X 35 cycles |
| Annealing | 61 | 45′′ | |
| Extension | 72 | 1′ | |
| Final extension | 72 | 5′ | 1 |
| Pause | 12 | ∞ | |

## 2.2 Analysis of pistil morphology and gene expression in different rice varieties subjected to heat stress conditions

This section will describe the methods used to characterise the expression of the selected genes in rice pistils pollinated under HS conditions, as well as the morphological characterisation of the pistils themselves.

### 2.2.1 Plant growth and heat stress treatment

Ten plants of eight different rice varieties (five cultivated in Portugal - Ariete, Caravela, Ceres, OP2115, and Teti - and three models - Kitaake, N22, and Nipp) were grown in a growth chamber, at 28°C/ 24ºC (light/dark), with a 12h light/12h dark photoperiod, and a light intensity of 500 µmol $m^{-2}$ $s^{-1}$. Just before the anthesis of the first flowers, five plants from each variety were transferred to another growth chamber with exactly the same conditions, except temperature, which in this chamber mimics HS, 38°C during the day and 32°C during the night, for 24-48 hours.

### 2.2.2 Isolation and observation of the rice pistils

Spikelets from the eight different rice varieties flowering under optimal and HS conditions were collected after anthesis and kept on ice. Subsequently, under a Leica MZ6 stereo microscope (Leica, Germany), the lemma was separated from the palea, and the pollinated pistils were isolated. The pistils were placed in Eppendorf tubes, cryopreserved with liquid

nitrogen, and stored at -80°C until RNA extraction. Three biological replicates, each one containing 25–30 pistils, were sampled for each rice variety and condition.

To observe differences in pistil morphology among the different varieties, isolated pistils were examined under a stereo microscope AXIO Zoom.V16 (Zeiss, Germany) and photos were taken.

### 2.2.3 RNA extraction from the pollinated pistils

Cell lysis of pistil samples was performed using a TissueLyser LT (Qiagen, Netherlands) operated at 50 Hz for 1 min. RNA extraction was accomplished using the Direct-Zol RNA Miniprep kit (Zymo Research, USA), following the manufacturer's instructions. To verify if the extraction was successfully executed, the RNA was quantified with a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) and a 1% agarose gel was run with 2.5 µL of the extracted RNA sample mixed with TriTrack DNA Loading Dye (6X) (Thermo Fisher Scientific, USA).

### 2.2.4 DNAse treatment

To eliminate residual genomic DNA, RNA purification was performed using TURBO DNA-free kit (Thermo Fisher Scientific, USA), following the instructions provided. To verify the effectiveness of the treatment, a PCR was performed for the housekeeping gene *P5CS*. The 20 µL reaction contained 4 µL of enzyme buffer, 1.6 µL of $MgCl_2$, 0.4 µL of dNTPs (10 mM), 0.8 µL of each primer (10 µM) (**Table 2.8**), 0.16 µL of GoTaq G2 Flexi DNA polymerase, 1 µL of RNA, and free-nuclease water. The *P5CS* amplification program is described in **Table 2.9**, and it was carried out in a thermocycler. The DNA from a WT plant was used as a negative control and water was used as positive control. A 1% agarose gel was used to run the PCR product (*P5CS* amplicon), and if bands were visible, the purification procedure was repeated for the sample in question.

**Table 2.8 - Primers used for *P5CS* amplification by PCR.**

| Primer | Sequence | Length (bp) | Tm (ºC) | %GC |
|---|---|---|---|---|
| *P5CS*_FW | AAGATGGAAGATTGGCTTTGGGCAG | 25 | 68.6 | 48.0 |
| *P5CS*_RV | TCATGCCTCCTCTACCTACACGAGA | 25 | 68.9 | 52.0 |

Table 2.9 - PCR program to amplify *P5CS*.

| Reaction Step | Temperature (ºC) | Time | Cycles |
|---|---|---|---|
| Initial denaturation | 95 | 5′ | 1 |
| Denaturation | 95 | 45′′ | X 35 cycles |
| Annealing | 58 | 45′′ | |
| Extension | 72 | 1′ | |
| Final extension | 72 | 5′ | 1 |
| Pause | 12 | ∞ | |

### 2.2.5 cDNA synthesis

cDNA was synthesised using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Switzerland), following the instructions provided. The protocol consisted of two reactions. In the first step, corresponding to primer annealing, RNA was mixed with oligo dT (deoxythymidine) primers and water to a final volume of 5.7 µL. The RNA concentration used was 1000 ng/µL. However, in less concentrated samples, the maximum amount of RNA volume (5.2 µL) was used. In the second step, reverse transcription was carried out using the RNA-primer mixture as a template, in a reaction of 10 µL. Following synthesis, cDNA samples were quantified using the Nanodrop and diluted to a final concentration of 10 µL/mL.

### 2.2.6 Calibration curve and optimisation of qPCR primers

To evaluate primer efficiency and obtain a normalised value for gene expression, the primers were optimised by using different primer concentrations and a cDNA calibration curve. The cDNA calibration curve was obtained after adding 5 µL of each of the twenty-four most concentrated cDNA samples, followed by serial dilutions at ratios of 1/2, 1/5, 1/10, 1/25, 1/50, 1/100, and 1/200. The 10 µL reverse quantitative PCR (qPCR) reaction contains 500, 1000, or 1500 nM of forward and reverse primers (20 µM) (**Table 2.10**), 5 µL of SYBR Green I Master mix (Roche, Switzerland), 2.5 µL of cDNA mix from the calibration curve, and nuclease-free water. Reactions were carried out using the Light Cycler 480 system (Roche, Switzerland), with an extension temperature of 60°C.

In case of nonspecific products or low amplification of the target gene due to its low expression, the previous steps were repeated, adjusting the extension temperature to 62°C or increasing the cDNA concentration to the maximum amount possible.

Primer efficiency was calculated by plotting the logarithm of the dilution factor against the corresponding Ct (cycle threshold) values of the calibration curve. With the slope obtained, the negative of its inverse was raised to 10. When this was not possible, the LinRegPCR software was used. The efficiencies close to a value of two were considered acceptable.

**Table 2.10 - Primers used for RT-qPCR analysis.**

| Primer | Sequence | Length (bp) | Tm (ºC) | %GC |
|---|---|---|---|---|
| *OsUBC2*_qPCR_FW | TTGCATTCTCTATTCCTGAGCA | 22 | 62.7 | 40.9 |
| *OsUBC2*_qPCR_RV | CAGGCAAATCTCACCTGTCTT | 21 | 63.4 | 47.6 |
| *OsPRXII*_qPCR_FW | AAGCCAAGGGTGTAGACGAC | 20 | 64.9 | 55.0 |
| *OsPRXII*_qPCR_RV | TATGACTTTGCCCACGCCTT | 20 | 65.3 | 50.0 |
| *OsWRKY55*_qPCR_FW | CGGAAGCCTATTCTTGGGGG | 20 | 65.4 | 60.0 |
| *OsWRKY55*_qPCR_RV | CTGACATTCGGTGCTGCAAG | 20 | 64.7 | 55.0 |
| *OsPLDalpha4*_qPCR_FW | GGCATCTGCTAACCTACCCG | 20 | 65.3 | 60.0 |
| *OsPLDalpha4*_qPCR_RV | ATTGATCGCCTTCTTCCCCAG | 21 | 65.3 | 52.4 |
| *OsWRKY53*_qPCR_FW_2 | GGGAACAACAAGCTGGAGGA | 20 | 65.2 | 55.0 |
| *OsWRKY53*_qPCR_RV_2 | TTCTTCATGGAGCAGCCGTT | 20 | 65.2 | 50.0 |
| *OsLEA17*_qPCR_FW | AGGCGACCAAGAACAAGCTG | 20 | 65.9 | 55.0 |
| *OsLEA17*_qPCR_RV | TTGAACTCCGTCGCCTTCTG | 20 | 65.3 | 55.0 |
| *OsYABBY7*_qPCR_FW | TGGAGCCTAACATCACCCAC | 20 | 64.8 | 55.0 |
| *OsYABBY7*_qPCR_RV | TCGCTTCTGCTGGATTCTGG | 20 | 65.2 | 55.0 |

### 2.2.7 Real-time qPCR for the housekeeping genes and genes of interest

qPCR was performed on pistil samples to analyse the expression of the housekeeping genes *OsUBC2* and *OsPRXII*, as well as the selected genes *OsWRKY55*, *OsPLDalpha4*, *OsWRKY53*, *OsYABBY7*, and *OsLEA17*. Each 10 µL reaction contains the optimised concentration of primers (**Table 2.10**), 5 µL of SYBR Green I Master mix, 2 µL of cDNA, and nuclease-free water. For each sample, two technical replicates were prepared, and water was used as a negative control. Reactions were carried out using the Light Cycler 480 system, with an extension temperature of 60°C or 62°C.

Gene expression levels were calculated after taking into account primer efficiency and averaging the two technical replicates. Normalisation was performed using the housekeeping genes' expression, as suggested by the Pfaffl comparison method [48].

Statistical analyses were performed using two-way ANOVA to evaluate the variation in gene expression under optimal and HS conditions in the different varieties. When significant effects were detected, post hoc test Šidák was applied. Differences were considered statistically significant at p-value < 0.05. All analyses were conducted using GraphPad Prism software.

## 2.3 Comparative yield analysis of rice varieties pollinated under optimal and heat stress conditions

### 2.3.1 Plant growth

To evaluate the heat tolerance of rice varieties cultivated in Portugal in comparison with the tolerant reference variety, N22, a yield assay was performed under optimal and HS conditions. To this end, twenty plants of each variety were grown in a growth chamber under control conditions, at 28°C/24°C (light/dark), with a 12h light/12h dark photoperiod, light intensity of 500 µmol $m^{-2}$ $s^{-1}$, and RH 60%. Before anthesis, ten plants of each variety were transferred to a second growth chamber with conditions that mimic HS, 38°C/ 32°C (light/dark), without changing the photoperiod, humidity, and light conditions, for 14 days. After this period, the panicles that experienced HS during anthesis were marked and the plants returned to control conditions until they were ready to harvest seeds.

### 2.3.2 Yield determination

When the rice plants complete their life cycle, panicles from each plant were harvested individually and stored in separate bags. Subsequently, yield was evaluated, under control and HS conditions, by counting the number of panicles, total number of spikelets, and filled seeds. From these data, fertility rate was calculated as the ratio of filled seeds to the total number of spikelets. In addition, the weight of 100 seeds was measured as an indicator of grain filling and to assess potential effects of HS on seed development. In the end, the mean of these values for each plant was calculated to determine the corresponding average for each variety.

# 3

# RESULTS AND DISCUSSION

## 3.1 Generation of rice plants knocked out for selected genes

Based on the integration of transcriptomic and database information, it was observed that *OsWRKY55* and *OsPLDalpha4* were downregulated in N22, the heat-tolerant rice variety, under HS conditions, and that they were mainly expressed in the pistils [25], [36]. Thus, they were selected as candidate genes for single-knockout in rice (Nipp genotype), aiming to increase HS tolerance at the fertilisation stage. Additionally, *OsWRKY53* gene was included due to its homology with *OsWRKY55* within the WRKY domain [38], its expression in the pistils [36], and the possibility of functional redundancy. Therefore, a double-knockout of both genes was also generated. To produce the edited plants, three CRISPR-Cas9 plasmids were constructed, two with single sgRNA for *OsWRKY55* and *OsPLDalpha4* genes (previously constructed) and one with double sgRNA for *OsWRKY53* and *OsWRKY55* genes (constructed during this MSc project). These three plasmids were introduced into Agrobacterium, which was subsequently used for rice transformation. The rice plants regenerated from the transformation process were genotyped (amplifying the hyg *hptII* marker gene by PCR) to verify the success of the rice transformation and the InDels produced by the CRISPR-Cas9 construct were analysed by sequencing after DNA amplification by PCR.

### 3.1.1 Assembly and validation of the double sgRNA CRISPR-Cas9 construct targeting *OsWRKY53* and *OsWRKY55* genes

Since the CRISPR-Cas9 plasmids for *OsWRKY55* and *OsPLDalpha4* were already constructed at the beginning of this MSc project, we only had to construct the plasmid with the two guides for *OsWRKY53* and *OsWRKY55*. This plasmid was generated using the Golden Gate

technology. First, the sgRNAs targeting *OsWRKY55* and *OsWRKY53* genes were cloned in L1P3 and L1P4 vectors, respectively, and the resulting plasmids were transformed in the *E. coli* strain DH5α [46], and plated on LB supplemented with ampicillin, IPTG, and X-gal. As expected, only bacteria harbouring the respective construct and, consequently, resistant to ampicillin, were able to grow. Through blue-white screening, three positive colonies were identified, and after growing them on liquid medium, plasmid DNA was extracted. After sequencing one representative colony for each construct, the results showed that the assembly of both vectors with their respective sgRNA was performed correctly. The plasmid L1 vectors carrying the individual sgRNAs were assembled in the final level 2 acceptor vector and introduced into *E. coli*, as described in the Materials and Methods. In this case, selection was carried out on spectinomycin-containing medium. Plasmid DNA from two positive colonies was analysed by restriction digestion using *Sal*I enzyme and by DNA sequencing. Gel electrophoresis results (**Figure 3.1**) confirmed the presence of the predicted digestion pattern (fragments of 8347, 4122, 1939, and 511 bps), despite faint and distorted bands. Those are caused by the small volume of buffer used during the gel electrophoresis. Furthermore, the sequencing results reconfirmed the correct assembly of the final L2_dgRNA_*WRKY55_WRKY53* construct.

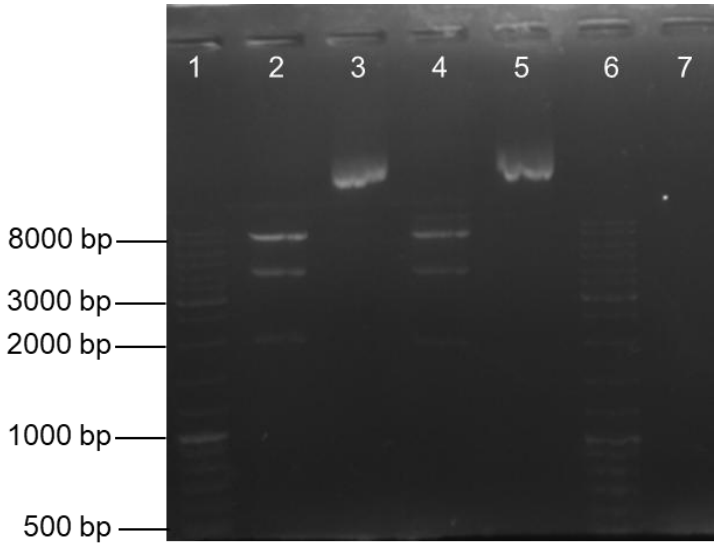


**Figure 3.1 - Restriction analysis of the final construct L2_dgRNA_*WRKY55_WRKY53*.** Plasmid DNA digestion was carried out with the *Sal*I enzyme and analysed in a 1% agarose gel electrophoresis. Lane 1 and 6: DNA ladder; Lane 2: digested L2_dgRNA_*WRKY55_WRKY53* plasmid DNA extracted from colony 1; Lane 3: undigested L2_dgRNA_*WRKY55_WRKY53* plasmid DNA extracted from colony 1; Lane 4: digested L2_dgRNA_*WRKY55_WRKY53* plasmid DNA extracted from colony 2; Lane 5: undigested L2_dgRNA_*WRKY55_WRKY53* plasmid DNA extracted from colony 2. The expected restriction pattern is 8347, 4122, 1939, and 511bp.

### 3.1.2 Agrobacterium transformation with the CRISPR-Cas9 constructs

To transform rice with the three CRISPR-Cas9 vector constructs designed to knockout *OsWRKY55, OsPLDalpha4,* and the double *OsWRKY53* and *OsWRKY55* genes, the vectors were introduced into *Agrobacterium tumefaciens*. And, to confirm the success of the transformations, a colony-PCR targeting a fragment of the *hptII* gene present in the construct was performed on colonies grown in rifampicin and spectinomycin containing medium. As a result, a ~750 bp fragment was expected to be obtained, indicating correct amplification of the amplicon. **Figure 3.2** shows the analysis of the colony PCR for the Agrobacterium transformed with the L2_dgRNA_*WRKY55_WRKY53* construct, and the success is shown in the figure.

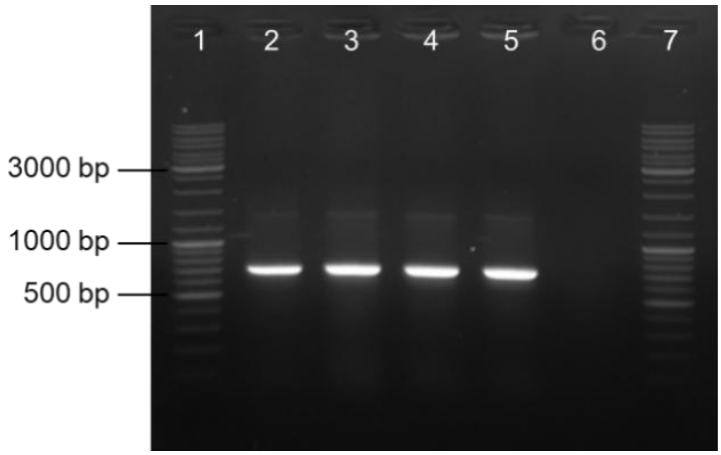


**Figure 3.2 - Analysis of the Agrobacterium tumefaciens colonies transformed with the L2_dgRNA_*WRKY53_WRKY55* vector.** A 750 bp fragment from the *hptII* gene, present in the L2_dgRNA_*WRKY53_WRKY55* vector, was amplified by PCR analysis and resolved in a 1% agarose gel electrophoresis. Lanes 1 and 7: DNA ladder; Lanes 2 to 5: Four colonies of Agrobacterium tumefaciens transformed with the L2_dgRNA_*WRKY55_WRKY53* construct; Lane 6: Negative control - water.

### 3.1.3 Rice transformations to knockout *OsWRKY55*, *OsPLDalpha4*, and *OsWRKY53*/*OsWRKY55* genes

To better understand the molecular mechanisms underlying HS tolerance in rice and trying to develop more tolerant varieties, rice was transformed with CRISPR-Cas9 constructs aiming to perform single-knockouts of the *OsWRKY55* and *OsPLDalpha4* genes and double-knockouts of *OsWRKY53* and *OsWRKY55*. In **Figure 3.3**, it is possible to observe the gene

structures and the designed sgRNA target sites, where the corresponding edits will occur, while **Figure 3.4** shows the different steps involved in rice transformation.

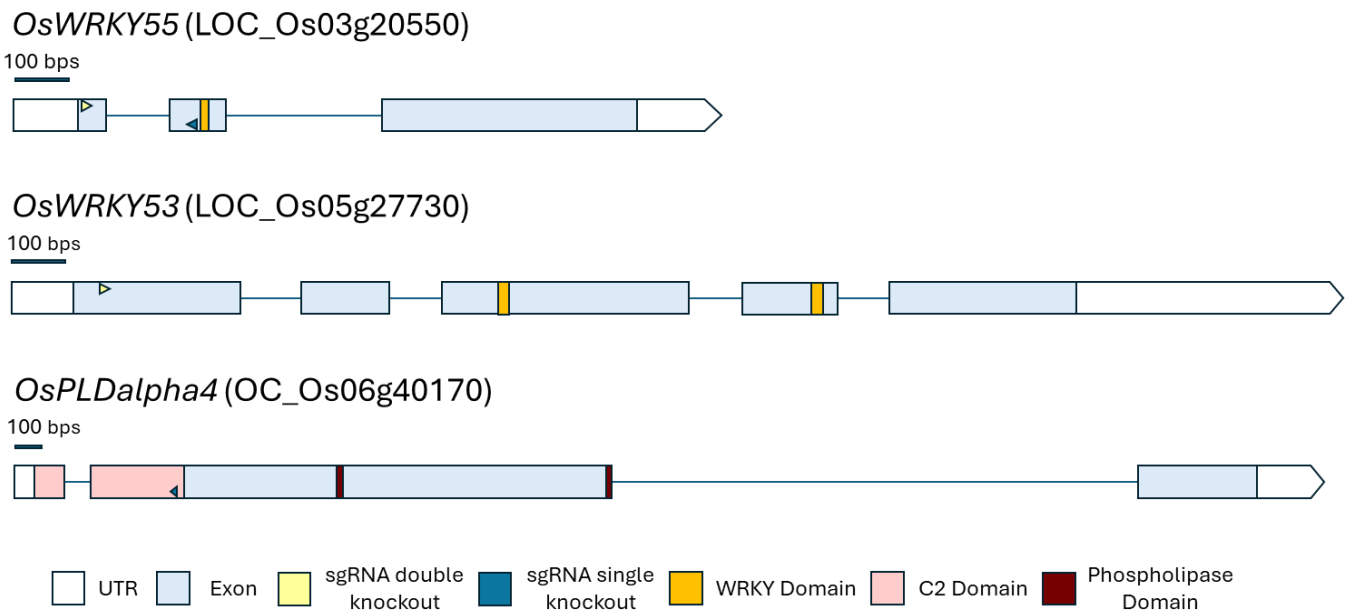


**Figure 3.3 - Schematic representation of the gene structure of *OsWRKY55*, *OsWRKY53*, and *OsPLDalpha4*.** The scale used for the *OsPLDalpha4* gene is half of the scale used for the *OsWRKY* family genes. Data from [25], [35].

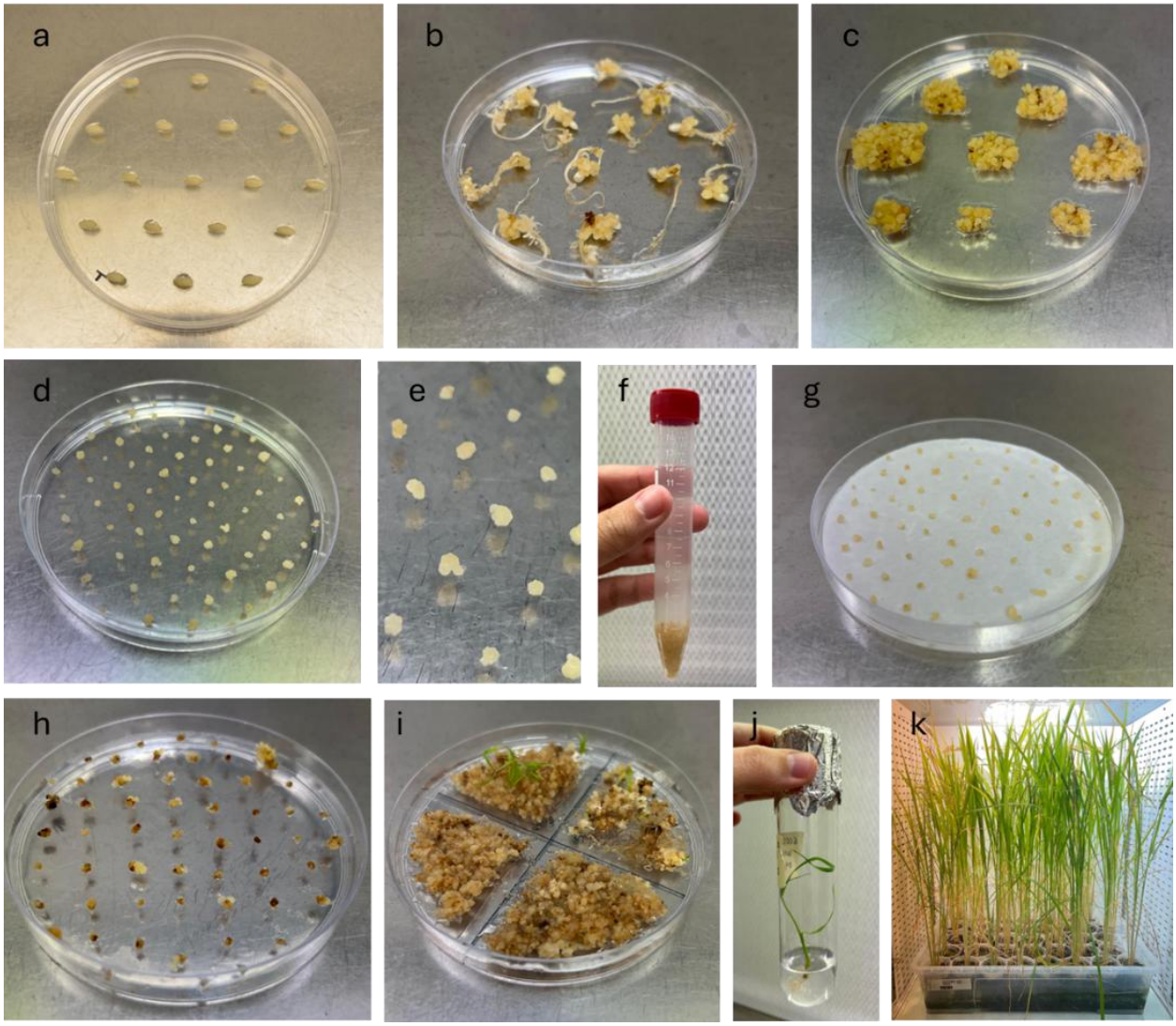


**Figure 3.4 - Representation of the different steps involved in rice transformation mediated by *Agrobacterium tumefaciens*.** (a) Disinfected rice seeds germinating in *callus* induction medium (CIM). (b) Dedifferentiated rice seeds in CIM, presenting three structures: seed, *scutella*, and radicle. (c) *Scutella* in CIM, after being separated from the other structures. (d) Embryogenic *callus* separated from the *scutella* in CIM. (e) Embryogenic *callus* seen with higher definition. (f) Embryogenic *callus* incubated in *Agrobacterium tumefaciens* for transformation. (g) Embryogenic *callus* after transformation in co-cultivation medium solid (CCMS) with a paper filter. (h) *Callus* transformed in selection medium (SM). (i) Proliferation of hygromycin-resistant *callus* in regeneration medium (RM) and shoot regeneration. (j) Growth and rooting of plantlets transformed in plant development medium (PDM). (k) Transformed plants growing in soil.

As summarised in **Table 3.1**, a total of nine transformations were performed: two for *OsWRKY55* (line E), three for *OsPLDalpha4* (line F), and four for the double-knockout *OsWRKY53* and *OsWRKY55* (line G). Overall, the transformations were successful, except for the F003 (*OsPLDalpha4*) and G003 (*OsWRKY53*/*OsWRKY55*), since no *calli* were able to grow

after the selection medium (SM) steps. The most likely explanation for this could be the incorrect selection of embryogenic *calli* [49].

**Table 3.1 - Summary of the rice transformations carried out to generate plants with the *OsWRKY55*, *OsPLDalpha4*, or *OsWRKY53* and *OsWRKY55* knockout genes.**

| Gene | Transformation | Number of *calli* after selection | *Calli* lines that regenerated into plants | Transformed c*alli* lines |
|---|---|---|---|---|
| *OsWRKY55*_KO | E001 | 44 | 17 | 15 |
| | E002 | 21 | 7 | 5 |
| Total | | 65 | 24 | 20 |
| *OsPLDalpha4*_KO | F001 | 47 | 9 | 9 |
| | F002 | 40 | 4 | 4 |
| | F003 | 0 | 0 | 0 |
| Total | | 87 | 13 | 13 |
| *OsWRKY53*_ *OsWRKY55*_KO | G001 | 16 | 5 | 3 |
| | G002 | 2 | 1 | 1 |
| | G003 | 0 | 0 | 0 |
| | G004 | 43 | 7 | 7 |
| Total | | 61 | 13 | 11 |

From these transformations, fifty-two plants were regenerated for *OsWRKY55* from twenty-four different *calli*/lines, twenty-five plants for *OsPLDalpha4* from thirteen *calli*/lines, and twenty-nine plants for the double-knockout of the *OsWRKY53* and *OsWRKY55* genes from thirteen *calli*/lines.

To confirm whether the regenerated plants were transformed, a PCR was performed to amplify a ~750 bp fragment of the *hptII* gene using genomic DNA extracted from the T0 plant leaves. **Figure 3.5** illustrates an example of a gel electrophoresis run to analyse rice seedlings regenerated from various transformations. This analysis allowed us to conclude that twenty out of the twenty-four lines regenerated for *OsWRKY55* were transformed, all thirteen lines regenerated for *OsPLDalpha4* were transformed, and eleven of the thirteen lines regenerated for *OsWRKY53/OsWRKY55* were transformed. These results also showed the presence of six lines with a false positive result, meaning that they were able to grow in a medium containing hyg

even though they had not been transformed. Some explanations for this unexpected growth are the insufficient selection pressure in SM, EIM, and PDM media due to the low concentration or degradation of hyg [50]. Another possible justification is that if the selected *callus* is too large, only a portion of its cells may become transformed, allowing hyg metabolization.

The positive T0 plants (showing *hptII* amplification) were transferred to pots with soil, where they grew until maturity.

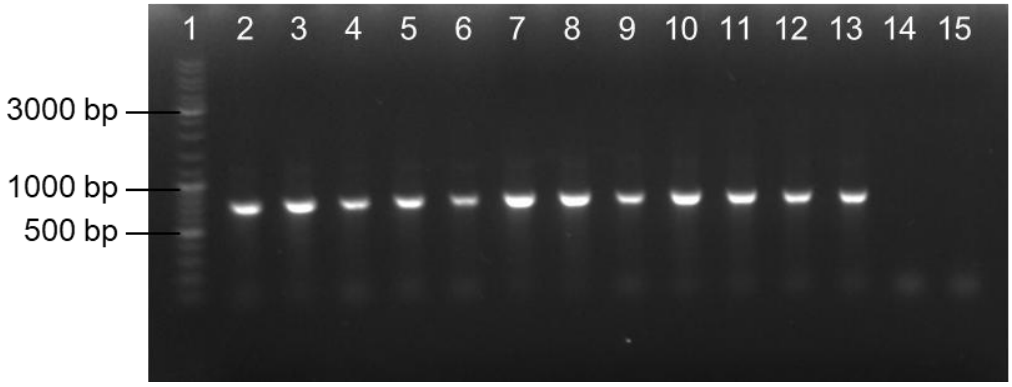


**Figure 3.5 - PCR analysis of rice seedlings regenerated after transformation with Agrobacterium. A fragment of the *hptII* gene was amplified by PCR and analysed in a 1% agarose gel electrophoresis.** Lane 1: DNA ladder; Lanes 2 to 13: rice seedlings generated from different transformations; Lanes 14 and 15: water and a previously tested negative sample (rice seedling not transformed). The expected band size for the *hptII* fragment is 750 bp.

To investigate whether the *hptII*-positive T0 plants were edited in the selected genes, we designed primers for PCR amplification flanking the edit-site. The PCR-product for each T0 plant was sequenced (**Tables 3.2 and 3.3**). Through the edits, it is possible to observe that the mutations occurred in the expected CRISPR-Cas9 cut-site, downstream of the PAM sequence [51].

In the case of single-KO lines, most plants exhibited biallelic edits, although some of them retained a WT genotype. For double-KO lines, mutations were detected in both target genes in almost all lines, except for two that remained WT for both loci. Additionally, we obtained one line that carried an *OsWRKY55* allele without edits, and another harboured a 3-nucleotide deletion in one of the *OsWRKY53* alleles. A similar 3-nucleotide deletion was also observed in one of the *OsWRKY55* alleles from a different line. Overall, the mutations were able to cause a frameshift, leading to protein truncation and impaired function, thereby enabling gene functional studies under optimal or HS conditions. In contrast, lines carrying 3-nucleotide

deletions maintain the reading frame, and the resulting proteins may remain functional. These specific lines might be useful in future generations to select plants carrying a functional *OsWRKY55* allele while maintaining a non-functional *OsWRKY53*, thus enabling the study of the *OsWRKY53* gene's contribution to heat tolerance.

Regarding the presence of non-edited plants with a WT sequence among the transformants, several factors may explain this observation. These include potential off-target cleavage events due to DNA sequences that resemble the target site, leading to mutations outside the intended genomic region, DNA repair after the Cas9 strand breaks that restores de WT sequence, or a lack of editing in the specific embryogenic *calli* that gave rise to the regenerated plant [52]–[54]. It is also possible that editing may occur in subsequent generations if the Cas9/sgRNA cassette remains integrated in the genome [54].

Our analysis also revealed that some plants regenerated from the same *calli* exhibited distinct InDels between them. This could be explained by the unintentional mixing of *calli* from different lines during the process of transferring to new medium plates. However, a more plausible explanation is that the editing performed by Cas9 may have occurred after the callus began to grow and regenerate, thus leading to different editing events among cells within the same *callus* [55]. Despite this, multiple independent and correctly edited lines were successfully obtained for each target gene.

As demonstrated in previous studies, gene knockouts are a valuable approach for elucidating gene function and assessing their role in specific stress responses [41], [56], [57]. Although CRISPR-Cas9 mediated knockout have not yet been reported for *OsWRKY55* and *OsPLDalpha4* genes, it has been well documented for the *OsWRKY53 gene*. The knockout of *OsWRKY53* has been shown to enhance bacterial resistance due to cell membrane thickening [56], as well as to improve tolerance to salinity [56] and cold [41]. Therefore, the edited lines generated in this study can be useful to assess the contribution of these genes to thermotolerance and the double-knockout may show multi-stress resistance.

**Table 3.2 - Edits obtained for the single-knockout of *OsWRKY55* and *OsPLDalpha4* genes.** The hyphen denotes a nucleotide deletion, while the N denotes a nucleotide insertion.

| Gene | Number of transformed plants | sgRNA target region | InDel | Zygosity | Resulting protein |
|---|---|---|---|---|---|
| *OsWRKY55*_KO | 2 | ACCTTTGCGCCGCACAACGA | 0 (WT) | Homozygous | Original (210 aa) |
| | 27 | ACCTTTGCGCCGCACA**N**ACGA | +1/+1 | Homozygous | Truncated (49 aa) |
| | 5 | ACCTTTGCGCCGCACA**N**ACGA | +1/+1 | Heterozygous | Truncated (49 aa) |
| | 2 | ACCTTTGCGCCGCACA**N**ACGA<br>ACCTTTGCGCCGCACA**NN**ACGA | +1/+2 | Heterozygous | Truncated (49 or 36 aa) |
| *OsPLDalpha4* | 3 | ACAAGAATAAGCTCCCCCAC | 0 (WT) | Homozygous | Original (832 aa) |
| | 2 | ACAAGAATAAGCTCCCC**N**CAC | +1/+1 | Heterozygous | Truncated (598 aa) |
| | 2 | ACAAGAATAAGCTCCCC**N**CAC | +1/+1 | Homozygous | Truncated (598 aa) |
| | 1 | ACAAGAATAAGCTCCC**--**AC<br>ACAAGAATAAGCTCCC**-**CAC | -2/-1 | Heterozygous | Truncated (466 or 597 aa) |
| | 1 | ACAAGAATAAGCTCCCC**N**CAC<br>ACAAGAATAAGCTCCC**-**CAC | +1/-1 | Heterozygous | Truncated (598 or 466 aa) |
| | 1 | ACAAGAATAAGCTCCCC**N**CAC<br>ACAAGAATAAGCTCCCC**NN**CAC | +1/+2 | Heterozygous | Truncated (598 or 467 aa) |
| | 1 | ACAAGAATAAGCTCCCC**--**C<br>ACAAGAATAAGCTCCCC**N**CAC | -2/+1 | Heterozygous | Truncated (466 or 598 aa) |
| | 3 | ACAAGAATAAGCTCCCC**-**CAC | -1/-1 | Homozygous | Truncated (466 aa) |
| | 1 | ACAAGAATAAGCTCCCC**--**C | -2/-2 | Homozygous | Truncated (466 aa) |

**Table 3.3 - Edits obtained for the double-knockout of *OsWRKY53* and *OsWRKY55* genes.** The hyphen denotes a nucleotide deletion, while the N denotes a nucleotide insertion.

| Gene | Number of transformed plants | Gene edited | sgRNA target region | InDel | Zygosity | Resulting protein |
|---|---|---|---|---|---|---|
| *OsWRKY53_OsWRKY55_*KO | 2 | *WRKY53* | GTGAACGACGTGATGAANCGG<br>GTGAACGACGTGATG--CGG | +1/-2 | Heterozygous | Truncated (40 or 39 aa) |
| | | *WRKY55* | GATTGATGCGGACTCGGNCAC<br>GATTGATGCGGACTCGGNNCAC | +1/+2 | Heterozygous | Truncated (49 or 14 aa) |
| | 1 | *WRKY53* | GTGAACGACGTGA----CGG<br>GTGAACGACGTGATGAACGG | -4/0 | Heterozygous | Truncated (202 aa) or Original (487 aa) |
| | | *WRKY55* | GATTGATGCGGACTCGG**N**CAC | +1/+1 | Heterozygous | Truncated (49 aa) |
| | 2 | *WRKY53* | GTGAACGACGTGAT---CGG<br>GTGAACGACGTGATGAANCGG | -3/+1 | Heterozygous | Truncated (40 aa) or 486 aa |
| | | *WRKY55* | GATTGATGCGGACTCGGNCAC | +1/+1 | Heterozygous | Truncated (49 aa) |
| | 2 | *WRKY53* | GTGAACGACGTGATGAACGG | 0 (WT) | Homozygous | Original (487 aa) |
| | | *WRKY55* | GATTGATGCGGACTCGGCAC | 0 (WT) | Homozygous | Original (214 aa) |
| | 1 | *WRKY53* | GTGAACGACGTGATGA-CGG<br>GTGAACGACGTGATGAA**N**CGG | -1/+1 | Heterozygous | Truncated (202 or 40 aa) |
| | | *WRKY55* | GATTGATGCGGACTCGG**N**CAC | +1/+1 | Homozygous | Truncated (49 aa) |
| | 1 | *WRKY53* | GTGAACGACGTGATGA-CGG<br>GTGAACGACGTGAT---CGG | -1/-3 | Heterozygous | Truncated (202 aa) or 486 aa |
| | | *WRKY55* | GATTGATGCGGACTCGG**N**CAC | +1/+1 | Heterozygous | Truncated (49 aa) |
| | 2 | *WRKY53* | GTGAACGACGTGA-------<br>GTGAACGACGTGA----CGG | -7/-4 | Heterozygous | Truncated (200 or 201 aa) |
| | | *WRKY55* | GATTGATGCGGACTCGG**N**CAC | +1/+1 | Homozygous | Truncated (49 aa) |
| | 2 | *WRKY53* | GTGAACGACGTGATGAA**N**CGG<br>GTGAACGACGTGA-----GG | +1/-5 | Heterozygous | Truncated (40 or 37 aa) |
| | | *WRKY55* | GATTGATGCGGACTCGG**N**CAC<br>GATTGATGCGGACTC---AC | +1/-3 | Heterozygous | Truncated (49 aa) or 213 aa |
| | 1 | *WRKY53* | GTGAACGACGTGATGAA**N**CGG | +1/+1 | Homozygous | Truncated (40 aa) |
| | | *WRKY55* | GTGAACGACGTGATGAA**N**CGG<br>GTGAACGACGTGA-----GG | +1/+2 | Heterozygous | Truncated (49 or 36 aa) |
| | 1 | *WRKY53* | GTGAACGACGTGATGAA**N**CGG | +1/+1 | Homozygous | Truncated (40 aa) |
| | | *WRKY55* | GATTGATGCGGACTCGGNCAC<br>GATTGATGCGGA-------- | +1/-10 | Heterozygous | Truncated (49 or 9 aa) |

## 3.2 Analyses of the pistil morphology and gene expression in the different rice varieties

In parallel to the generation of edited plants for the genes of interest, it was decided to characterise the heat tolerance of a number of rice varieties cultivated in Portugal - Ariete, Caravela, Ceres, OP2115, and Teti - and model varieties - Kitaake, N22, and Nipp. With this study, we expected to understand whether there is a correlation between heat tolerance among the different varieties and differences in pistil morphology and the genetic expression profile of the selected HS-responsive genes. In addition to studying genes less expressed in the tolerant variety N22 and or with downregulation behaviour in HS conditions and mainly expressed in pistils - *OsWRKY55*, *OsPLDalpha4*, and *OsWRKY53* - it was decided to include genes that are upregulated in the same conditions and tissue - *OsLEA17* and *OsYABBY17*.

### 3.2.1 Morphological pistil traits associated with potential heat stress tolerance in rice varieties

To assess potential morphological differences that may influence heat tolerance, among the different varieties under study, pistils were isolated and examined under an AXIO Zoom.V16 stereo microscope. However, it is important to note that these data are preliminary, as no technical replicates or morphometric measurements were performed. **Figure 3.6** shows that pistils from the different varieties are quite distinct from each other, presenting different sizes of styles and stigmas (length and width). For example, it can be observed that Ariete, Ceres, and Teti have a larger style. The opposite is true for N22 and Kitaake varieties, which have a shorter distance between the stigma and the ovary. Regarding stigmas, it is clear that the varieties with the longest stigmas are Ariete, OP2115, and Teti, while Caravela, Nipp, and N22 have the shortest stigmas. As for width, it is smallest in the Caravela, N22, and OP2115 varieties.

Although few studies have addressed the impact of female reproductive organ morphology on yield under HS conditions, stigma exsertion is recognised as a trait with potential effects on fertilisation and appears to be associated with longer styles and stigma length, combined with reduced stigma width [32], [33]. Nevertheless, this impact can be positive or negative depending on the time of day when HS occurs. In the case of HS at night, the absence of strong radiation and typically higher humidity makes this trait advantageous, as it increases surface area and prolongs the time pollen can be received, improving pollination and fertilisation. However, during daytime HS, the same trait becomes detrimental [17], [31]. High temperatures

combined with consequent desiccation, oxidative damage, and reduced stigma receptivity can cause asynchrony between stamens and pistils, resulting in decreased fertilisation [17], [31].

Global warming tends to cause rapid and continuous increases in both daytime and nighttime temperatures. Nevertheless, while daytime HS causes visible and acute sterility events, nighttime HS causes a chronic and widespread decrease in yield. This occurs because energy that would be available for grain filling is used, thus decreasing grain quality and yield, even when fertilisation was successful [17], [18]. Therefore, nighttime HS can be more detrimental to yield.

As our study was carried out under conditions of daytime and nighttime HS, and as nighttime HS appears to have a greater influence on yield, varieties with characteristics related to stigma exsertion (longer styles and stigma length, and reduced stigma width) may have a higher yield. Thus, considering that Ariete and Teti have large styles and long stigmas, while OP2115 has long stigmas and a small stigma width, these observations suggest these three varieties may be more heat-tolerant under the conditions used in our HS assay. However, yield analysis under HS in these varieties is needed to validate our hypothesis.

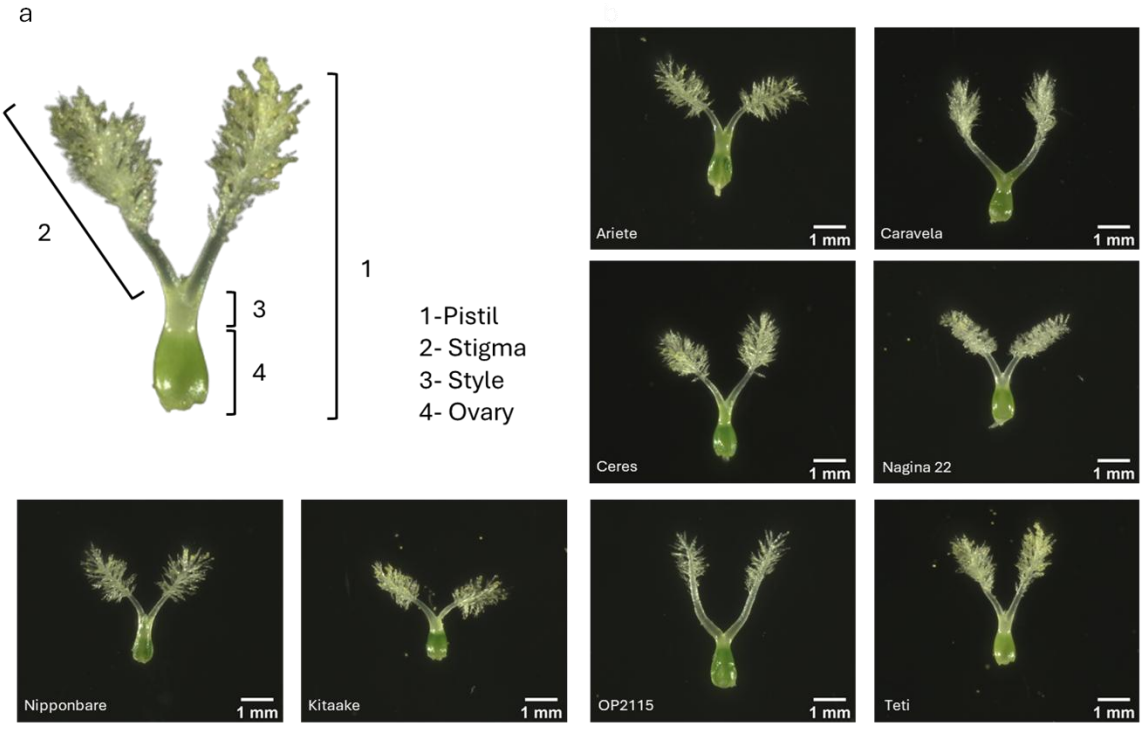


**Figure 3.6 - Pistil morphology in different rice varieties.** (a) Schematic representation of the different structures of the pistil. (b) Magnified image of the pistils isolated from different rice varieties, showing the structures of the pistil. Scale bar, 1 mm.

### 3.2.2 Expression of heat-stress responsive genes in different rice varieties

To evaluate the expression of the selected HS-responsive genes in different varieties under HS and optimal conditions by reverse transcription-quantitative PCR (RT-qPCR), primers optimisation was first performed to ensure specific and efficient amplification. Following primer optimisation, RT-qPCR analyses were performed using cDNA samples from pistils, pollinated under optimal or HS conditions, and the resulting data were analysed.

#### 3.2.2.1 Optimisation of primer pairs for RT-qPCRs

To optimise RT-qPCR primers for the selected genes, a calibration curve was performed using serial dilutions of cDNA from rice pistils (1/2, 1/5, 1/10, 1/25, 1/50, 1/100, and 1/200), and three primer concentrations (500, 1000, or 1500 nM) for each pair of primers. Primer performance was evaluated based on the Cts and the melting curve [58]. Regarding the Cts, it is expected that when the dilution is increased by half, there will be an increase of 1 Ct. In the case of the melting curve, the aim is to obtain a single, narrow peak, which means that the amplification was specific to the target (**Figure 3.7 a**). When non-ideal results were obtained (**Figure 3.7 b**), either the melting temperature was increased from 60°C to 62°C or the amount of cDNA was adjusted (in case of low Cts).

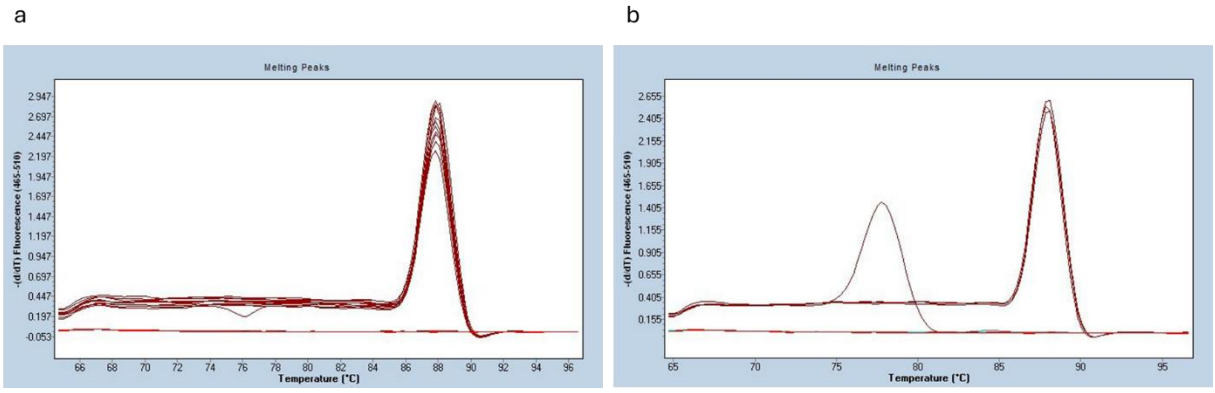


**Figure 3.7 - Example of melting curves obtained during primer optimisation**. (a) Melting curve showing specific product amplification. (b) Melting curve showing non-specific product amplification.

To obtain the efficiency of the primers, a graph was plotted between the log of the dilution and the Cts, and the negative of the inverse value of the slope was raised to 10. When insufficient data points were available, the LinRegPCR software was used. The conditions that allowed for the highest efficiency and specificity were selected for subsequent experiments.

The results of the efficiencies obtained for the different genes according to temperature and primer concentration are shown in **Table 3.4**.

For the *OsWRKY55* gene, it was possible to obtain a proper primer optimisation for all the different concentrations and the melting curve shows only one curve, indicating that the amplification was specific. Thus, it was decided to work with a primer concentration of 1000 nM, since the efficiency was the highest, comparable to that observed at 500 nM.

The optimisation of primers for the *OsPLDalpha4* gene did not work as expected. Since the expression of this gene is low, the Ct values were very high and there was an inconsistent amplification. Because of this, with a melting temperature of 60°C, it was only possible to have a proper amplification at a concentration of 1500 nM, being the only concentration that provided sufficient data points to construct a calibration curve. The melting curve also showed the presence of a specific product, leading to the selection of this concentration. However, during the qPCR to determine the expression of the gene in the different varieties, multiple nonspecific products were detected in the melting curve, contrary to initial results. To address this problem, qPCR was repeated using an annealing temperature of 62ºC, without checking the efficiency of the primers at this temperature. Under these conditions, amplification specificity improved, confirming the suitability of the designed primers. Primer efficiency was then re-analysed at 1500 nM with a melting temperature of 62°C. As it was not possible to obtain sufficient data points for the calibration curve, it was decided to determine the efficiency of the RT-qPCR run using the LinRegPCR software. Although the amplification efficiency was relatively low (80,1%), the gene was specifically and consistently amplified in all biological replicates of the varieties. Therefore, it was decided to continue with this pair of primers.

For the *OsWRKY53* gene, proper amplification was only successful using an annealing temperature of 62°C, for which the melting curve showed a specific amplification. Regarding primer concentration, it was chosen 1000 nM, since the efficiency was high and the melting curve showed a more uniform peak for all samples.

Regarding the *OsYABBY7* gene, its amplification was successfully performed at a temperature of 60°C. Amplification was specific and efficiency was very high at all primer concentrations. Therefore, a primer concentration of 500 nM was used, as its efficiency was the highest.

Finally, regarding the *OsLEA17* gene, it was only possible to have a good amplification using an annealing temperature of 62ºC and a primer concentration of 1000 nM. The reason for this could be related to the low expression of the gene since the Ct values were too high.

**Table 3.4 - Primer efficiencies obtained for the different genes according to temperature and primer concentration.** The chosen conditions are marked in bold.

| Gene | Temperature (ºC) | Primer concentration (nM) | Efficiency |
|---|---|---|---|
| *OsWRKY55* | **60** | 500 | 1.89 (94.5%) |
| | | **1000** | **1.89 (94.5%)** |
| | | 1500 | 1.80 (90.1%) |
| *OsPLDalpha4* | 60 | 1500 | 1.93 (97.0%) |
| | **62** | **1500** | **1.60 (80.1%)** |
| *OsWRKY53* | **62** | 500 | 1.90 (95.5%) |
| | | **1000** | **1.97 (98.7%)** |
| | | 1500 | 1.99 (99.7%) |
| *OsYABBY7* | **60** | **500** | **2.04 (102.1%)** |
| | | 1000 | 1.80 (90.1%) |
| | | 1500 | 1.96 (97.9%) |
| *OsLEA17* | **62** | **1000** | **1.87 (93.3%)** |

#### 3.2.2.2 Gene expression analysis by RT-qPCR

With the aim of studying the variation in the expression of the genes of interest (*OsWRKY55*, *OsPLDalpha4*, *OsWRKY53*, *OsYABBY7*, and *OsLEA17*) in the pistils of different rice varieties, pollinated under optimal and HS conditions, RT-qPCR was performed. By comparing the expression profiles of each variety under study with the heat-sensitive variety, Nipp, and the heat-tolerant variety, N22, we expect to infer how the expression profile of these genes is associated with the heat tolerance of these varieties. This approach is also expected to provide insights into which of the Portuguese cultivated varieties are more likely to exhibit enhanced tolerance under HS. **Figure 3.8** presents the expression of the different genes across the different varieties subjected to HS conditions

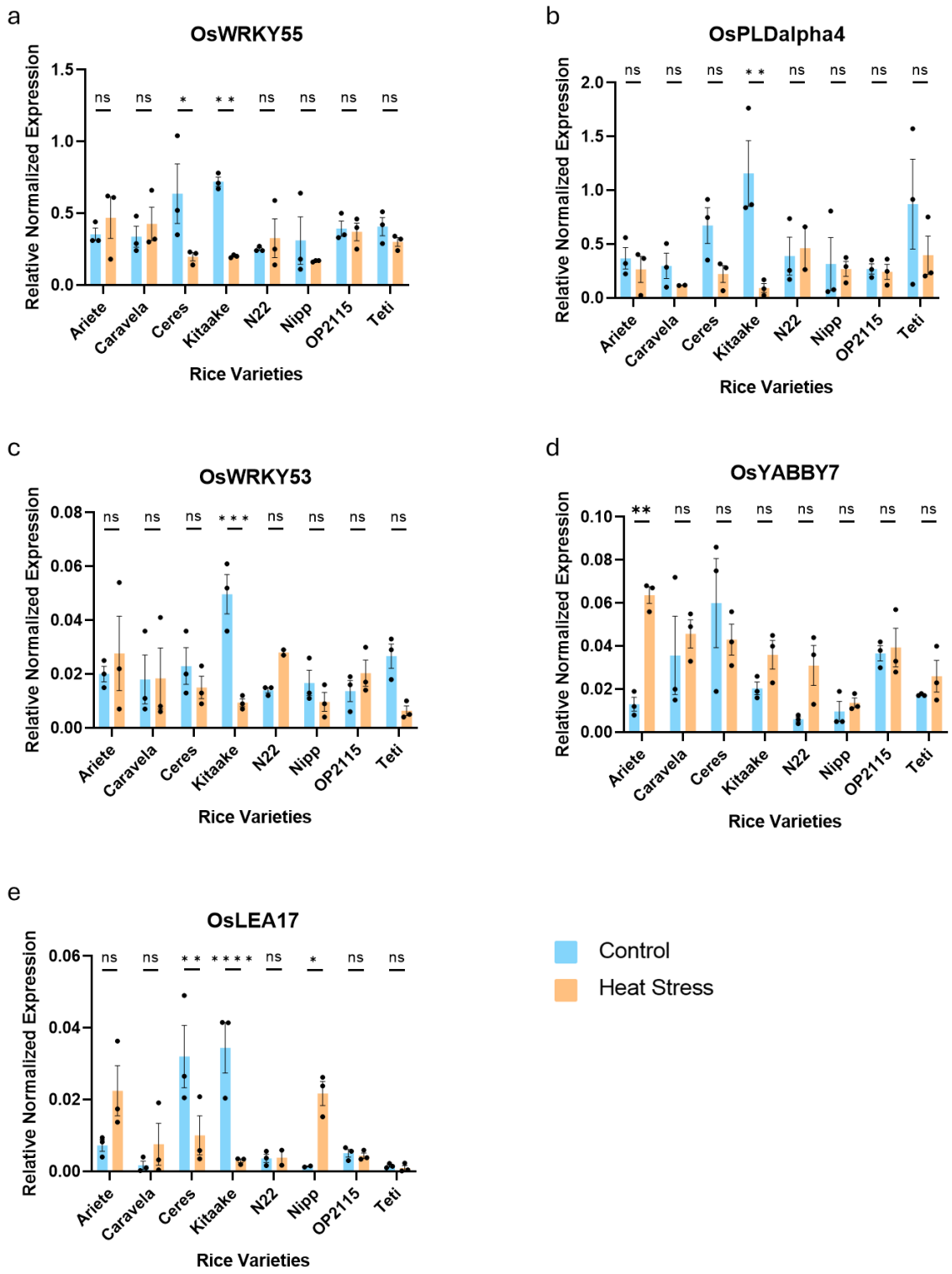


**Figure 3.8 - Relative expression of each gene of interest in different rice varieties under optimal conditions and under heat stress.** *OsPRXII* and *OsUBC2* were used as housekeeping genes and the relative expression was calculated via the Pfaffl method. The statistical analysis was performed using two-way ANOVA with a p-value < 0.05. Error bars represent the standard error of the mean (SEM) from three biological replicates (n=3). (a) Analysis of *OsWRKY55* gene expression. (b) Analysis of *OsPLDalpha4* gene expression. (c) Analysis of *OsWRKY53* gene expression. (d) Analysis of *OsYABBY7* gene expression. (e) Analysis of *OsLEA17* gene expression.

#### 3.2.2.2.1 Regulation of *OsWRKY55*, *OsPLDalpha4*, and *OsWRKY53* genes in pistils pollinated under heat stress

Regarding *OsWRKY55*, the transcript level for this gene varies among the different varieties (**Figure 3.8 a**) and significant downregulations in response to HS occurred only in Ceres and Kitaake, while Nipp, OP2115, and Teti show a tendency for downregulation, but without statistical significance. As for the Ariete, Caravela, and N22 varieties, overall, no significant change in *OsWRKY55* expression was observed.

As for the *OsPLDalpha4* gene, a detailed analysis of each variety (**Figure 3.8 b**) reveals that in Kitaake this gene is negatively regulated, showing statistically significant results, and that in Ceres and Teti it shows the same trend. The remaining varieties show no significant changes in their expression.

Regarding the expression of the *OsWRKY53* gene (**Figure 3.8 c**), there is a decrease in expression in Kitaake and a tendency for the same in Ceres and Teti. The remaining varieties, Ariete, Caravela, N22, Nipp, and OP2115, show no significant alterations in *OsWRKY53* expression.

Kitaake proved to be the variety whose transcript level is high and shows downregulation in these three genes under HS. This is very different from what is observed for N22, which shows low transcript levels under optimal conditions (as compared with Kitaake) and no change in expression in response to HS. Thus, Kitaake may be one of the most heat-sensitive varieties, which is suggested by previous studies [59]. The same is true for Ceres, as it shows significant downregulation for the *OsWRKY55* gene and *OsPLDalpha4* also tends to be downregulated by HS.

#### 3.2.2.2.2 *OsWRKY55* and *OsPLDalpha4* genes do not seem to be regulated in N22 under heat stress

When comparing the results obtained for the *OsWRKY55* and *OsPLDalpha4* genes (**Figure 3.9**) with previously data obtained by the University of Porto (**Figure 1.4**) for the N22 and Nipp varieties, notable discrepancies were observed. For *OsWRKY55*, prior data suggested that the overall expression in N22 would be lower than in Nipp, with downregulation under HS in N22 and stable expression in Nipp. In contrast, the results obtained indicate similar overall expression levels in both varieties, with no significant alterations in expression in N22 and downregulation in Nipp under HS conditions. Regarding *OsPLDalpha4*, it was a lower expression was previously observed in N22 compared to Nipp, with N22 showing stable expression under HS and Nipp exhibiting a strong decrease. In the current study, overall expression was

similar between the two varieties, with no significant alterations in expression in N22 and Nipp under HS (**Figure 3.9**). A plausible explanation for the discrepancies observed between our results and the ones from Porto likely lies in the number of biological replicates and the resulting statistical robustness. In the results from Porto, only a single biological replicate was analysed, which limits the reliability of the data and increases the potential error. In contrast, the present study included three biological replicates, providing greater statistical validity. However, some variability among replicates was still observed and the exclusion of the outliers would compromise statistical strength. Therefore, future studies should incorporate a larger number of biological replicates to improve accuracy of gene expression analyses.

Despite these differences, both genes remain suitable targets for knockout aimed at improving heat tolerance, as their general tendency among the different rice varieties is to maintain their expression or to be downregulated under HS. It should be noted that high variance in the data, particularly for N22 and Nipp, may contribute to an incorrect data analysis and underscores the need for more biological replicates and statistical validation in future analyses. Using other varieties with similar expression patterns, for example Kitaake, to generate knockouts could also be very interesting, allowing for a deeper understanding of gene function.

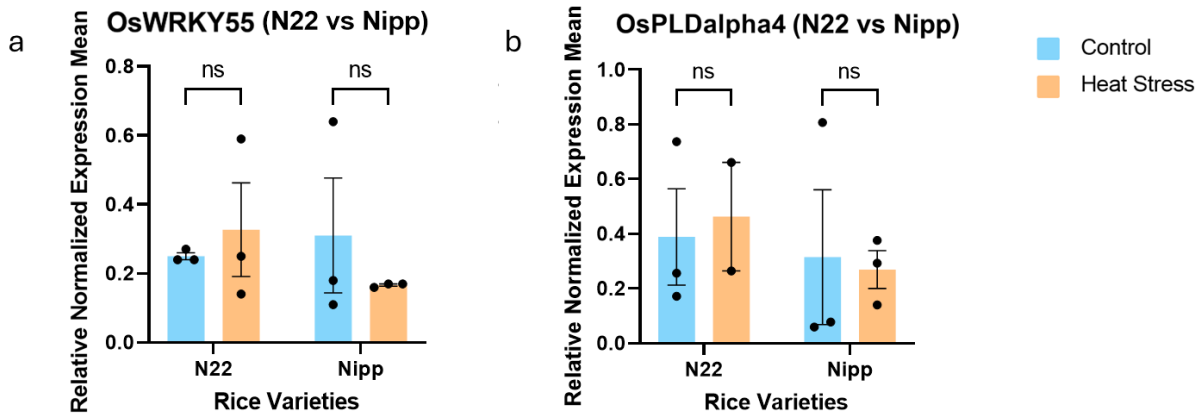


**Figure 3.9 - Relative gene expression for *OsWRKY55* and *OsPLDalpha4* in the pollinated pistils of Nagina 22 and Nipponbare subjected to heat stress.** The relative expression was calculated via the Pfaffl method. The statistical analysis was performed using t-tests with a p-value < 0.05. Error bars represent the standard error of the mean from three biological replicates (n=3). (a) Analysis of *OsWRKY55* gene expression. (b) Analysis of *OsPLDalpha4* gene expression.

#### 3.2.2.2.3 *OsYABBY7* gene is upregulated under heat stress

**Figure 3.8 d** shows that *OsYABBY7* is significantly upregulated in the pistils of Ariete pollinated under HS and the trend is followed in Caravela, Kitaake, and N22. In contrast, in Ceres there is a tendency to a downregulated, and the remaining varieties show no significant changes in expression. As for the *OsLEA17* gene, a similar analysis shows that *OsYABBY7* is significantly downregulated in Ceres and Kitaake (**Figure 3.8 e**). In Nipp, the opposite situation is observed, with significant upregulation under HS and a tendency towards the same in Ariete. The remaining varieties did not show significant changes.

The overall gene upregulation trend that several varieties showed for the expression of *OsYABBY7* under HS, including the high HS-tolerant N22, indicates that this gene can be useful for increasing the heat tolerance of rice through its overexpression [60]. This type of technique to increase tolerance has already been successfully implemented, namely, overexpression of heat shock proteins, which, as expected, are upregulated in situations of HS. Furthermore, considering the results obtained, Ariete may be one of the most tolerant varieties, since the *OsYABBY7* expression is upregulated, following the trend of N22. Regarding *OsLEA17*, no conclusions can be drawn about how its expression is altered by HS, as no consistent results were obtained.

## 3.3 Yield assay for different rice varieties subjected to heat stress at anthesis

To evaluate the heat tolerance at anthesis of the different varieties under study, Ariete, Caravela, Ceres, Kitaake, N22, Nipp, OP2115, and Teti, we performed a HS assay to assess yield under these conditions. This experiment also enabled the characterisation of their agronomic traits under control situations and served to test the hypotheses proposed regarding possible tolerance associated with the gene expression and pistil morphology observed. In this assay, plants were grown under optimal temperature conditions (28°C during the day and 24°C at night) and, immediately before anthesis, they were subjected to either the same conditions or subjected to HS for 15 days (38°C during the day and 32°C at night), and kept under these conditions for 15 days, making sure that all flowers/pistils were pollinated under HS.

### 3.3.1 Agronomical parameters of the different rice varieties under study grown and pollinated under optimal temperature conditions

In this assay, it was possible to characterise the agronomic traits of the different rice varieties grown and pollinated under optimal temperature conditions, through the average panicle number per plant, average number of spikelets per plant and filled seeds per plant, and fertility rate. (**Table 3.5**). Additionally, **Figure 3.10** illustrates the area occupied by 100 seeds of each variety in a circle, which allows for a relative assessment of the size and shape of the seeds. Thus, larger circles correspond to larger seed size.

Analysis of the agronomic performance under optimal temperature conditions revealed a clear variability among the different rice varieties (**Table 3.5**). In terms of panicle number, the model variety N22 exhibited the highest value. However, this did not correspond to a greater number of filled seeds per plant, as Kitaake outperformed it in this parameter. Ariete, Ceres, OP2115, and Teti had only three panicles, with Ceres showing the lowest total number of spikelets per plant, as well as the lowest fertilisation rate. Regarding fertility rate, Kitaake and N22 presented the highest values, while Ceres clearly displayed the lowest. Overall, these results indicate that Kitaake and N22 have the best agronomic performance, whereas Ceres has the least advantageous.

**Table 3.5 - Agronomic characterisation of the varieties under study in control conditions.** Data are presented as mean ± standard deviation (SD), from seven to eleven biological replicates (n=7-11).

| Variety | Average number of panicles | Average total spikelets per plant | Average filled seeds per plant | Average fertility rate (%) | Average weight of 100 seeds (g) |
|---|---|---|---|---|---|
| Ariete | 3 ± 1.2 | 103 ± 81.9 | 100 ± 72.9 | 92.8 ± 29.55 | 2.674 ± 0.1523 |
| Caravela | 4 ± 1.2 | 184 ± 54.0 | 173 ± 55.3 | 89.9 ±11.70 | 3.099 ± 0.1508 |
| Ceres | 3 ± 1.6 | 66 ± 55.6 | 39 ±39.8 | 62.8 ± 27.43 | 3.037 ± 0.2335 |
| Kitaake | 7 ± 1.1 | 292 ± 69.8 | 283 ± 62.5 | 95.5 ± 4.85 | 2.411 ± 0.0857 |
| Nagina 22 | 9 ± 2.5 | 295 ± 80.7 | 263 ± 77.6 | 94.9 ± 6.73 | 1.600 ± 0.0951 |
| Nipponbare | 7 ± 0.9 | 320 ± 96.1 | 282 ± 84.9 | 86.5 ± 4.95 | 2.013 ± 0.0287 |
| OP2115 | 3 ± 1.5 | 142 ± 50.0 | 128 ± 47.9 | 84.8 ± 15.36 | 3.498 ± 0.1478 |
| Teti | 3 ± 0.6 | 83 ± 25.7 | 64 ± 23.5 | 78.3 ± 8.28 | 2.519 ± 0.3796 |

Concerning seed morphology, significant differences can be seen between these rice varieties under study (**Figure 3.10**). Kitaake, N22, and Nipp seem to produce the smallest seeds, while OP2115 and Ceres had the largest. In terms of shape, OP2115 displayed a distinctly elongated shape, contrasting with the more rounded seeds of Kitaake. These results are consistent with the weight of 100 seeds, since varieties with larger seeds had a higher weight and the opposite was observed for varieties with smaller seeds.


**Figure 3.10 - Representation of the seeds morphology in different rice varieties.** Scale bar, 1 cm.

### 3.3.2 Nagina 22 exhibits the highest heat tolerance compared to all other tested varieties

The objective of this assay was to assess yield under HS conditions during anthesis, in order to investigate the impact of high temperatures on the reproduction of different rice varieties. By calculating the fertility rate and the weight of 100 seeds, it is possible to understand the extent of damage caused by HS during flowering and whether seed filling was affected. From the results obtained, we expected to identify the most heat-tolerant varieties and, eventually, to be able to correlate HS tolerance (although in a preliminary exercise) with gene expression pattern and pistil morphology. Contrary to our expectations, most rice varieties exposed to HS stress failed to produce viable seeds, resulting in complete infertility and null yield. Only Caravela, N22, Nipp, and Teti produced some viable seeds, and among these, only N22 consistently showed an average fertility rate above 4% in the plants analysed. This outcome

indicates that the applied stress was too severe and exceeded the tolerance threshold of most varieties.

Comparison with previous studies helps explain these results. in Wu C. *et al.* (2021), N22 exposed to HS, day/night temperatures of 33.3ºC/28.4ºC and 34.6ºC/30.9ºC for 7 days, retained 69.1% fertility [61]. Similarly, in Jagadish S. V. K. *et al.* (2010), N22 subjected to 6 hours during the day at 38ºC for two days during anthesis maintained 71% fertility [30]. In addition, exposure *japonica* varieties to daytime and nighttime HS (41°C/31°C) for over 6 days caused a 61.1% reduction in fertility [62], while 6h daily during the day over 14 days of exposure to 38ºC/27ºC led to only 2.1% fertility in IR64 [63]. These comparisons indicate that the stress applied in the present assay was too severe, particularly due to higher nighttime temperatures and long exposure to the stress, which explains the results of this assay.

Under optimal conditions, N22 exhibited superior agronomic performance compared to other varieties, as reflected in its high number of spikelets per plant, combined with high fertility and consistent seed set. When subjected to HS, its fertilisation rate was markedly affected, as shown in **Table 3.6**. A slight reduction was observed in the average weight of 100 seeds, and as previously noted, the most pronounced effect was on fertility, which decreased by approximately 80% under HS. Despite this substantial reduction, N22 maintained a fertility rate of 16%, whereas all other varieties exhibited sterility. This result highlights the exceptional thermotolerance of N22, consistent with previous studies reporting its stable reproductive performance under very high temperatures [25], [30], [61].

Thus, although the stress conditions applied in this assay were extremely severe, N22 remained as the only variety capable of producing viable seeds, confirming its status as a model for thermotolerance studies in rice [25].

**Table 3.6 - Agronomic characterisation of the varieties under study in heat stress conditions.** Data are presented as mean ± SD, from seven to ten biological replicates (n=7-10).

| Variety | Average number of panicles | Average total spikelets per plant | Average filled seeds per plant | Average fertility rate (%) | Average weight of 100 seeds (g) |
|---|---|---|---|---|---|
| Ariete | 4 ± 0.8 | 52 ± 14.5 | 0 | 0 | - |
| Caravela | 3 ± 0.7 | 108 ± 33.4 | 6 ± 7.6 | 3.3 ± 7.83 | 2.889 ± 0.1813 |
| Ceres | 2 ± 0.8 | 81 ± 52.1 | 0 | 0 | - |
| Kitaake | 6 ± 1.3 | 223 ± 62.5 | 0 | 0 | - |
| Nagina 22 | 7 ± 1.1 | 207 ± 69.0 | 32 ± 14.6 | 16.3 ± 8.08 | 1.478 ± 0,2458 |
| Nipponbare | 7 ± 1.1 | 369 ± 68.6 | 1 ± 6.3 | 0.1 ± 1.65 | 1.700 ± 0.0578 |
| OP2115 | 2 ± 1.4 | 95 ± 53.7 | 0 | 0 | - |
| Teti | 3 ± 0.6 | 116 ± 46.5 | 0 ± 1.9 | 0 ± 1.59 | - |

# 4

# CONCLUSIONS AND FUTURE WORK

Heat stress (HS) represents a major threat to rice production, especially when it occurs during anthesis, as fertilisation and yield are severely affected. Based on literature and database analyses, several pistil-expressed genes that were downregulated under HS in the tolerant variety Nagina 22 (N22) were initially selected. Their expression was further analysed in N22 and Nipponbare (Nipp) under optimal and HS conditions. From this analysis, *OsWRKY55* and *OsPLDalpha4* were chosen due to their reduced or consistently lower expression in N22 compared to Nipp. Additionally, *OsWRKY53* was selected for its homology within the WRKY domain to *OsWRKY55* and possible redundancy in the pathways related to HS. In this study, based on the selected genes, genome editing was successfully employed to generate CRISPR-Cas9 mediated single-knockout mutants for *OsWRKY55* and *OsPLDalpha4* genes and double-knockout mutants for *OsWRKY53*/*OsWRKY55* genes, producing truncated proteins with predicted loss of function. Thus, twenty transformed lines were obtained for the single-knockout of OsWRKY55, thirteen for the single-knockout of *OsPLDalpha4*, and eleven for the double-knockout of *OsWRKY53*/*OsWRKY55.* These lines represent valuable tools for elucidating the molecular mechanisms underlying heat tolerance in rice, as well as for a deeper functional characterisation of these genes.

In addition, the expression of HS-responsive and pistil-expressed genes (*OsWRKY55*, *OsPLDalpha4*, *OsWRKY53, OsYABBY7,* and *OsLEA17*) was evaluated across different rice varieties grown in Portugal - Ariete, Caravela, Ceres, OP2115, and Teti - and in control varieties - Kitaake, N22, and Nipp. It was observed that Kitaake and Ceres are the varieties that presented a significant or tendency for downregulation in the *OsWRKY55*, *OsPLDalpha4*, and *OsWRKY53* genes, while the expression of these genes in the tolerant variety N22 is much lower in both control and HS conditions and their expression remains unchanged. Thus, these two varieties

may be sensitive, which is partially confirmed by the study from Botelho *et al.,* (2025), which indicates that Kitaake is sensitive to heat [59]. It can also be concluded that Ariete may be more tolerant, since the expression of the *OsYABBY7* gene is upregulated in this variety, following the trend observed in N22. Regarding the *OsLEA17* gene, it is not possible to draw conclusions about the most tolerant variety, since there was no change in the expression of N22 and the results of the other varieties were not consistent. Furthermore, the expression profile of N22 and Nipp under HS was different from what was expected. Thus, this study did not allow to draw relevant conclusions about the association of gene expression patterns with HS tolerance. However, the knockout mutants generated may still have an advantage on HS tolerance since the general tendency of *OsWRKY55* and *OsPLDalpha4* is to maintain their gene expression or to be downregulated under HS.

Analysis of pistil morphology showed considerable variability among the different varieties and, based on the literature, it was possible to identify the varieties most likely to have stigma exsertion, a characteristic associated with heat tolerance during the night HS, known for being the most detrimental. This identification was made based on the characteristics associated with this trait - longer styles and stigma length, combined with reduced stigma width. Therefore, as Ariete, OP2115, and Teti varieties exhibit these characteristics, it can be hypothesised that they may have a natural tolerance to heat due to possibly exhibiting stigma exsertion.

The yield assay aimed to investigate the different rice varieties, both the agronomic performance under optimal temperature conditions and the HS tolerance at anthesis, thus revealing their level of HS tolerance. Under optimal temperature conditions, Kitaake seems to have the best agronomic performance, as it had the highest number of full seeds and the highest fertility rate. In contrast, Ceres was the worst performer, with the lowest number of full seeds and the lowest fertility rate. However, due to the severity of HS, grain filling was only obtained in variety N22, which led to the conclusion that this is the most tolerant rice variety among all those tested, reinforcing the relevance of this variety as a model for thermotolerance studies. Overall, this study enabled the combination of molecular, morphological, and agronomic data to advance both fundamental and applied knowledge on rice HS tolerance. Ultimately, it can be affirmed that there was a meaningful contribution toward enhancing rice productivity, thereby supporting the sustainability of global food security.

Future studies should focus on the phenotypic validation of the generated mutant lines under control and HS conditions. These analyses should assess agronomic parameters such as the total number of panicles per plant, the total number of filled seeds per plant, the fertility

rate, and the weight of 100 seeds. In addition, physiological and molecular traits should be evaluated under optimal and HS conditions, including pistil morphology, pollen viability, pollen tube elongation, photosynthetic efficiency, hormonal content, and reactive oxygen species (ROS) responses. Such characterisation will provide a comprehensive understanding of the mechanisms underlying the role of the selected genes in HS response. If enhanced tolerance is confirmed, these knockouts could be transferred to commercial cultivars such as Caravela, the first rice variety produced entirely in Portugal for over 30 years, thereby combining scientific advances with potential commercial applications.

Regarding the characterisation of pistil morphology of varieties cultivated in Portugal, as well as their gene-expression profiles for the selected HS-responsive genes, these analyses need to be repeated as the results and conclusions obtained are preliminary. As for the analysis of pistils, future studies should include biological replicates of at least three pistils per variety and perform accurate measurements of the length of the stigmas and styles and the width of the stigmas. Concerning the gene expression studies, it was observed that the RT-qPCR results showed high variance, which ultimately compromised the statistical analysis. Therefore, it is necessary to repeat this experiment using more biological replicates to achieve more meaningful results. It is also necessary to repeat the yield assay, subjecting the plants to HS at anthesis, but at this time with less severe HS conditions. Thus, it must be carried out with a nighttime temperature below 32°C, for approximately 6 days, with only 6 hours of daily exposure to stress.

Together, these future analyses will allow an accurate functional characterisation of the candidate genes, provide a deeper understanding of the pistil morphology and its influence on HS tolerance, and supporting the application of gene-editing techniques to develop a resilient rice variety adapted to the emerging challenges of global warming

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# 5

# APPENDICES

**Table 5.1 - Composition of the different media used for the different steps of rice transformation.**

| Media composition | CIM | CCML | CCMS | SM | EIM | RM | PDM |
|---|---|---|---|---|---|---|---|
| N6 Macro I | 100 mL/L | - | - | - | - | - | - |
| N6 Macro II | 100 mL/L | - | - | - | - | - | - |
| R2 Macro I | - | 100 mL/L | 100 mL/L | 100 mL/L | - | - | - |
| R2 Macro II | - | 100 mL/L | 100 mL/L | 100 mL/L | - | - | - |
| MS Macro I | - | - | - | - | 100 mL/L | 100 mL/L | 50 mL/L |
| MS Macro II | - | - | - | - | 100 mL/L | 100 mL/L | 50 mL/L |
| MS FeNa-EDTA | 10 mL/L | - | - | - | 10 mL/L | 10 mL/L | 5 mL/L |
| R2 FeNa-EDTA | - | 10 mL/L | 10 mL/L | 10 mL/L | - | - | - |
| B5 Vitamins | 1 mL/L | - | - | - | - | - | - |
| R2 Vitamins | - | 25 mL/L | 25 mL/L | 25 mL/L | - | - | - |
| LS Vitamins | - | - | - | - | 10 mL/L | 10 mL/L | 5 mL/L |
| B5 Micro | 1 mL/L | - | - | - | - | - | - |
| R2 Micro | - | 1 mL/L | 1 mL/L | 1 mL/L | - | - | - |
| MS Micro | - | - | - | - | 1 mL/L | 1 mL/L | 0.5 mL/L |
| Proline | 500 mg/L | - | - | - | - | - | - |
| Glutamine | 500 mg/L | - | - | - | - | - | - |
| CEH | 300 mg/L | - | - | - | - | - | - |
| Glucose | - | 10 g/L | 10 g/L | - | - | - | - |
| Sucrose | 30 g/L | - | - | 30 g/L | 30 g/L | 40 g/L | 10 g/L |
| Gelrite | 2.5 g/L | - | 7 g/L | 7 g/L | 7 g/L | 7 g/L | 2.5 g/L |
| 2,4-D | 2.5 mg/L | 2.5 mg/L | 2.5 mg/L | 2.5 mg/L | 2.5 mg/L | - | - |
| Acetosyringone | - | - | 1 mL/L | - | - | - | - |
| Cefotaxime | - | - | - | 1 mL/L | 1 mL/L | 1 mL/L | - |
| Vancomycin | - | - | - | 1 mL/L | 1 mL/L | 1 mL/L | - |
| Hygromycin | - | - | - | 1 mL/L | 2 mL/L | - | 0.5 mL/L |
| Coconut water | - | - | - | - | 100 mL/L | - | - |
| IAA | - | - | - | - | - | 0.5 mg/L | - |
| BAP | - | - | - | - | - | 0.3 mg/L | - |
| NAA | - | - | - | - | - | - | 0.05 mg/L |

Table 5.2 - Composition of the different nutrient solutions used to prepare media for rice transformation.

| Nutrient solutions composition (g/L) | $KNO_3$ | $(NH_4)SO_4$ | $KH_2PO_4$ | $MgSO_4 \cdot 7H_2O$ | $CaCl_2 \cdot 2H_2O$ | Myo-inositol | Thiamine HCl | Nicotinic acid | Pyridoxine HCl | $MnSO_4 \cdot H_2O$ | KI | $H_3BO_3$ | $ZnSO_4 \cdot 7H_2O$ | $CuSO_4 \cdot 5H_2O$ | $Na_2MoO_4 \cdot 2H2O$ | $CoCl_2 \cdot 6H_2O$ | $NH_4NO_3$ | $FeSO_4 \cdot 7H_2O$ | $Na_2EDTA \cdot 2H_2O$ | FeNaEDTA | $NaH_2PO_4 \cdot H_2O$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N6 Macro I | 8.3 | 4.63 | 4 | 1.85 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| N6 Macro II | - | - | - | - | 1.66 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| R2 Macro I | 40 | 3.3 | - | 2.46 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 3.12 |
| R2 Macro II | - | - | - | - | 1.46 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| MS Macro I | 19 | - | 1.7 | - | 3.7 | - | - | - | - | - | - | - | - | - | - | - | 16.5 | - | - | - | - |
| MS Macro II | - | - | - | - | 4.4 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| MS FeNEDTA | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1.25 | 0.177 | - | - |
| R2 FeNa-EDTA | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 3.67 | - |
| B5 Vitamins | - | - | - | - | - | 10 | 1 | 0.1 | 0.1 | - | - | - | - | - | - | - | - | - | - | - | - |
| R2 Vitamins | - | - | - | - | - | - | 0.04 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| LS Vitamins | - | - | - | - | - | 10 | 0.04 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| B5 Micro | - | - | - | - | - | - | - | - | - | 10 | 0.75 | 3 | 2 | 0.039 | 0.25 | 0.025 | - | - | - | - | |
| R2 Micro | - | - | - | - | - | - | - | - | - | 1.6 | - | 2.83 | 2.2 | 0.195 | 0.125 | - | - | - | - | - | - |
| MS Micro | - | - | - | - | - | - | - | - | - | 16.9 | 0.83 | 6.2 | 8.6 | 0.025 | 0.25 | 0.025 | - | - | - | - | - |