

RiceltUp : Understanding the heat stress mechanisms of *Oryza sativa*

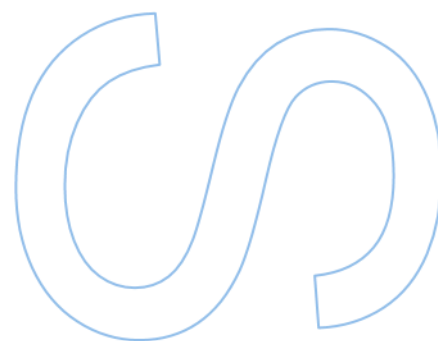
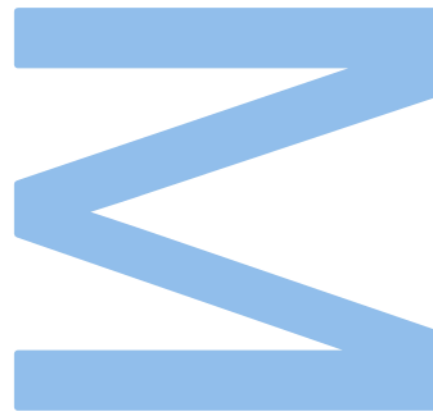
Ana Ventura Azevedo da Silva Bravo

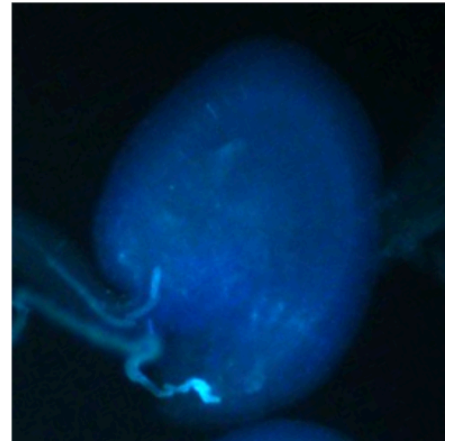
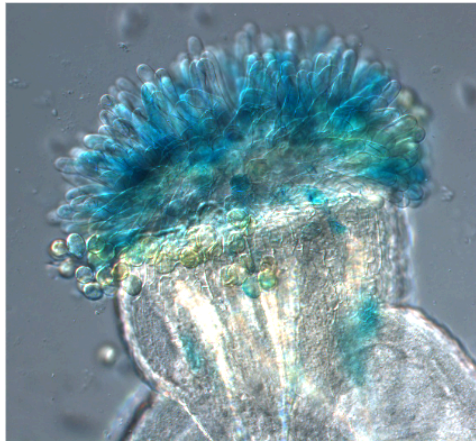
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RiceltUp : Understanding the heat stress mechanisms of *Oryza sativa*

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Resumo

As plantas são essenciais para os humanos. São uma fonte de alimento, fármacos, entre outros. O arroz (*Oryza sativa*) fornece cerca de 35-60% das calorias diárias a nível global, e estima-se que sejam cultivados mais de 164 milhões de hectares de arroz anualmente.

No entanto, a quantidade e qualidade do arroz são bastante afetados pelo calor. Por cada 1°C de aumento de temperatura, há um declínio de 6,2-10% na produção de arroz, sendo a fase reprodutiva a mais sensível a este stress. As consequências do stress por calor em inflorescências de arroz incluem desenvolvimento anormal do pólen, falta de deiscência das anteras, baixa receptividade do estigma, defeitos na germinação e crescimento do tubo polínico e desenvolvimento anormal do gametófito. Devido às alterações climáticas e ao aquecimento global, estes problemas vão se tornar cada vez mais frequentes, levando a um grande impacto na produção de alimentos.

Assim, este projeto teve por objetivo estudar o mecanismo molecular de tolerância ao stress por calor de *O. sativa* ao nível reprodutivo. Um RNA-seq realizado anteriormente em pistilos da variedade tolerante ao calor Nagina22 foi usado para selecionar a lista de genes candidatos que podem estar relacionados com o mecanismo de tolerância de *O. sativa*, e os seus ortólogos em *Arabidopsis thaliana* foram identificados. O nível de expressão dos genes selecionados em condições controlo e de calor foram validados por RT-qPCR. Uma linha de inserção de T-DNA para o gene de interesse foi selecionada e analisada por clareamento de síliquas e coloração de azul de anilina para caracterização do fenótipo reprodutivo em condições de controlo e de calor. Foram gerados constructos para linhas repórter e de sobreexpressão para *A. thaliana*, e constructos de sobreexpressão e Prime editing para arroz.

A análise da linha de inserção de T-DNA demonstrou um atraso significativo no crescimento do tubo polínico e uma diminuição da taxa de fecundação dos óvulos em condições de stress por calor. No entanto, a formação de sementes não foi comprometida neste mutante.

Este estudo fornece uma boa base de estudo para explorar como os genes candidatos atuam na tolerância ao calor. Embora o seu impacto preciso ainda não tenha sido totalmente esclarecido, estes resultados permitem direccionar investigações futuras.

Palavras-chave: Stress por calor, *Oryza sativa*, *Arabidopsis thaliana*, reprodução das plantas.

Abstract

Plants are fundamental to humans. They are a source of food and pharmaceuticals, among other things. Rice (*Oryza sativa*) provides about 35-60% of global daily calories to humans world-wide, and it is estimated that 164 million hectares of rice are cultivated annually.

However, the quantity and quality of rice are heavily affected by heat. For every 1°C increase in temperature, there's a decline in rice production of 6.2-10%, being the reproductive period the most sensitive one. The consequences of heat stress in rice inflorescences include abnormal pollen development, lack of anther dehiscence, low stigmatic receptivity, defects in germination and elongation of the pollen tube and abnormal gametophyte development. Due to climate change and global warming, these problems will become increasingly frequent, leading to a major impact on food production.

Therefore, this project aimed to study the *O. sativa*'s heat stress tolerance molecular mechanisms at the reproductive level. A previous RNA-seq performed on pistils from a heat-tolerant rice variety, Nagina22 was used to select a list of candidate genes that might be related to the heat stress mechanism of *O. sativa*, and their orthologues in *Arabidopsis thaliana* were identified. The expression level of the selected genes in control and heat stress conditions was validated by RT-qPCR. A T-DNA insertion line in *A. thaliana* was selected for the gene of interest, and was analysed by seed clearing and aniline blue staining for phenotypic characterization in control and heat stress conditions. Constructs for reporter and overexpression lines in *Arabidopsis*, and overexpression and prime editing lines in rice were generated.

The analysis of the T-DNA insertion line revealed a significant delay in the pollen tube growth and a lower ovule fecundation ratio in heat stress conditions. However, the seed formation was not compromised in this mutant.

The study lays a good foundation for exploring the involvement of the candidate genes in heat stress tolerance. Although their precise impact remains to be fully elucidated, the results highlight promising directions for future investigation.

Keywords: Heat stress, *Oryza sativa*, *Arabidopsis thaliana*, plant reproduction.

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List of Abbreviations

<i>AtACT8</i>	ACTIN8
<i>AtRUB1</i>	RELATED DO UBIQUITIN1
BB	BACTERIAL BLIGHT
BE	BASE EDITING
BP	BORDER PRIMER
CDS	CODING SEQUENCE
COL-0	COLUMBIA-0 ECOTYPE
CWIN	CELL-WALL INVERTASE
DIC	DIFFERENTIAL INTERFERENCE CONTRAST
DSB	DOUBLE-STRAND BREAKS
dsDNA	DOUBLE-STRANDED DNA
DEG	DIFFERENTIALLY EXPRESSED GENE
<i>EXTRNA</i>	EXTENSION RNA
gDNA	GENOMIC DNA
GFP	GREEN FLUORESCENCE PROTEIN
GUS	B-GLUCURONIDASE
HDR	HOMOLOGY-DIRECTED REPAIR
HM	HOMOZYGOUS
HS	HEAT STRESS
HSE	HEAT-SHOCK ELEMENTS
HSF	HEAT-SHOCK TRANSCRIPTION FACTORS
HSP	HEAT-SHOCK PROTEINS
HT	HETEROZYGOUS

lncRNAs	ENDOGENOUS LONG NONCODING RNAs
KIT	KITAKE
LB	LURIA BERTANI
LP	LEFT PRIMER
MT	MUTANT
N	NORMAL CONDITIONS
N22	<i>NAGINA22</i>
NHEJ	NON-HOMOLOGOUS END JOINING
NIPP	NIPPONBARE
NS	NON-SIGNIFICANT
<i>OsEF1</i>	EARLINESS 1
<i>OsGIF</i>	GRAIN INCOMPLETE FILLING 1
<i>OsPHR</i>	PHOSPHATE STARVATION RESPONSE1
<i>OsNABP</i>	NUCLEIC ACID BINDING PROTEIN
OV	OVULE
PAM	PROTOSPACER ADJACENT MOTIF
PBS	PRIMER BINDING SITE
PCD	PROGRAMMED CELL DEATH
PE	REVERSE TRANSCRIPTASE FUSED TO A Cas9 NICKASE
PEGRNA	PRIME EDITING GUIDE RNA
PG	POLLEN GRAIN
PT	POLLEN TUBE
RAM	ROOT APICAL MERISTEM
RAP-DB	RICE ANNOTATION PROJECT DATABASE
ROS	REACTIVE OXYGEN SPECIES

RP	RIGHT PRIMER
S	STIGMA
SAM	SHOOT APICAL MERISTEM
SB	SODIUM BORATE
SIGNAL	SALK INSTITUTE GENOMIC ANALYSIS LABORATORY
SOB	SUPER OPTIMAL BROTH
ssRNA	SINGLE-STRANDED RNA
ST	STYLE
TAE	TRIS-ACETATE-EDTA
TAIR	THE ARABIDOPSIS INFORMATION RESOURCE
TEDA	T5 EXONUCLEASE DNA ASSEMBLY
TT	TRANSMITTING TISSUES
WT	WILD TYPE
xCas9	<i>SpCAS9</i>
Xoo	XANTHOMONAS ORYZAE PATHOVAR ORYZAE

1. Introduction

1.1. Sexual reproduction in Angiosperms

Angiosperms, or flowering plants, are the most diverse group within the plant kingdom, comprising approximately 90% of the terrestrial plant species. It is estimated that this group includes around 374 000 species, distributed into 64 orders and 416 families (Christenhusz and Byng, 2016; Zuntini *et al.*, 2024). Angiosperms are essential for humans, as they constitute a primary source of food, like cereals, fruits and vegetables, medicinal compounds, resins, fibers, paper and other economically important products (Dubey and Prakash, 2021). The seeds are particularly important, not only because they contribute 60% of the global caloric intake, but also because they are indispensable for the propagation of crops and fruit species, being fundamental for agriculture (Fernández-Pascual *et al.*, 2019).

Studies suggest that Angiosperms first appeared in the Early Cretaceous, approximately 145-100 million years ago (Ma) and rapidly expanded and diversified, becoming the biggest group of terrestrial plants (Ramírez-Barahona *et al.*, 2020). This reproductive success is likely connected with the origin of flowers, which evolved from the reproductive cones of the Gymnosperms. Flowers are linked to many reproductive advantages, including a faster and more efficient life cycle, opportunity for gametic competition, endosperm as nutritional supply of the seeds, ovary protection, among others (Soltis *et al.*, 2019). Given the central role of these structures for the success of this group, they are also subject to a strong selective pressure, allowing them to acquire a wide range of morphological diversity over time (Sauquet and Magallón, 2018). However, even within this diversity, it is still possible to define a common structure to most angiosperms. They typically contain the reproductive structures, surrounded by modified sterile leaves that provide protection. Most flowers are composed of four floral organs: sepals, petals, stamens (consisting of filament and anther) and carpels. Inside the carpels are the ovules, attached to the ovary by the funiculus. Upon fertilization, the ovules develop into seeds (Irish, 2010).

Angiosperms have a haplodiplont life cycle, defined by the alternation between two multicellular generations: a diploid generation, composed by the sporophyte, and a haploid generation composed by the gametophyte. However, since the gametophytes

develop inside the sporophytic tissues, this alternation of generations is often inconspicuous. These plants are also heterosporous, meaning that the sporophytes produce two distinct types of spores. The male spores (microspores), are small, lightweight, nutrient-poor and produced in large quantities. They are produced in the anthers and optimized for dispersion and long-distance propagation. And the female spores (megaspores) that are produced in the ovule. They are larger and nutrient-rich, but produced in less quantity. Inside the microspores and megaspores, the microgametophytes and megagametophytes develop through a process known as gametogenesis. The gametophytes subsequently produce the gametes by mitosis (Fig.1) (Qiu *et al.*, 2012).

The megagametophyte develops within the ovules, located inside the ovary, itself enclosed within the carpel. The ovule contains a single megasporocyte ($2n$), that will originate four megaspores (n) by meiosis, three of those that are going to degenerate by Programed Cell Death (PCD). The functional megaspore undergoes three successive mitotic divisions, resulting in a total of eight haploid nuclei: three antipodal cells at the chalazal pole, two polar nuclei, one egg cell (oosphere) and two synergid cells with a filiform apparatus at the micropylar pole. Together, these structures form the embryo sac or female gametophyte (Yadegari and Drews, 2004; Palanivelu and Tsukamoto, 2012).

The microsporocytes ($2n$) undergo meiosis to form a tetrad composed of four haploid microspores (n). This meiotic division is also responsible for the transcriptomic reprogramming and cytoplasm remodulation, which eliminates all the residual transcripts from the diploid phase (Dickinson and Heslop-Harrison, 1977). A layer of callose is subsequently deposited around the tetrad and between the microspores (De Storme and Geelen, 2013) Once the exine starts to be formed, the tapetum cells start to secrete calase (beta-1,3-glucanase), which degrades the callose surrounding the tetrad. (Scott *et al.*, 2004). After being released from the callose wall, the microspores undergo an asymmetrical division, resulting in two different daughter cells: a large vegetative cell and a small generative cell. The vegetative cell constitutes the majority of the pollen cytoplasm, has decondensed chromatin and organelles, and is responsible for mechanically supporting the sperm cells and accumulating reserves for the rapid growth of the pollen tube (PT) (Butel and Köhler, 2024). This cell also accumulates osmoprotectants, such as proline, that protect the membranes and proteins from desiccation-induced damage (Pacini *et al.*, 2006; Mattioli *et al.*, 2018).

The generative cell is small, has condensed chromatin and maintains its proliferative capacity, undergoing a second division to form the two male gametes (Lalanne and Twell, 2002; Russel and Jones, 2015). This second division can occur before or after pollen maturation, and it can take place within the anther as in *Arabidopsis thaliana*, or during the pollen tube growth (Borg *et al.*, 2009; Lord e Russell, 2002).

The pollen wall is formed during the pos-meiotic development of the microspore. This structure plays a critical role in protecting the microgametophyte as well as mediating the communication with the stigma surface following pollination (Jiang *et al.*, 2013). This structure consists of three distinct layers: the intine, primarily composed of pectin, cellulose, hemicellulose and proteins; the exine, with a non-uniform distribution across the pollen surface and primarily composed of sporopollenin; and the pollen coat, primarily composed of lipids and proteins, which is only completed during the final stages of microgametogenesis, when remnants of tapetal cells are deposited onto the pollen surface (Zhang and Yang, 2016; Quilichini *et al.*, 2015). The pollen wall has a dual origin, involving both the sporophytic and gametophytic phases, since the tapetum contributes for the exine and pollen coat biosynthesis, whereas the pollen grain is responsible for the formation of the intine (Ariizumi and Toriyama, 2011; Gibalová *et al.*, 2009).

The mature pollen grain eventually reaches the stigma, where it is recognized as compatible, adheres to the stigmatic surface and is hydrated, germinates and forms the PT. The rapid Ca^{2+} influx marks the site of the PT emergence. The pollen coat lipids and pistil-secreted factors as brassinosteroids and sulfated azadecalins promote this germination (Iwano *et al.*, 2004; Qin *et a.*, 2011; Vogler *et al.*, 2014). Then, digestive enzymes such as O-fucosyltransferase, ceramidase and extensions will facilitate the PT penetration into the female tissues (Smith *et al.*, 2018; Chen *et al.*, 2015). During its elongation, the PT forms an inner callose wall, and periodically forms callose plugs that block older regions of the tube, maintaining cytoplasmic contents concentrated near the growing tip (Franklin-Tong, 1999). The PT transports the two male gametes through the transmitting tissue (TT), a specialized tissue connecting the stigma, style and ovules. The TT supports PT nutrition and growth until directional cues guide it toward the ovule (Pereira *et al.*, 2015; Yadegari and Drews, 2004). The PT guidance can be divided in two phases: a sporophytic phase, independent of the female gametophyte, where the PT grows through the intracellular spaces of the stigma, guided by molecular signals produced by this structure; and a gametophytic phase, dependent of signals produced

by the female gametophyte, that guides the PT along the funiculus toward the micropyle, where it enters the embryo sac through one of the two synergid cells (Ray *et al.*, 1997; Higashiyama *et al.*, 2003; Pereira *et al.*, 2016). Once inside the embryo sac, the PT ceases its growth, ruptures and releases the two male gametes. The synergid cell that receives the PT degenerates, and the second synergid undergoes PCD, preventing polyspermy (Higashiyama *et al.*, 2001; Dresselhaus and Franklin-Tong, 2013; Dresselhaus, 2006). One of the male gametes fuses with the oosphere and the other fuses with the central cell, originating the zygote and the endosperm, respectively. These tissues develop into a seed that germinates to form a new sporophyte, thereby completing the plant life cycle.

One of the most important developmental transitions during the reproduction phase of the angiosperms is the anthesis, consisting in the transition from floral bud to an open flower. The anthesis is marked by both morphological changes - such as petal expansion and alterations in flower color and scent - and metabolic changes - including increased synthesis and accumulation of secondary metabolites, essential for pollinators attraction and TP guidance. Key reproductive events occur during anthesis, including anther dehiscence, pollination and pollen tube germination and elongation (Arshad *et al.*, 2017).

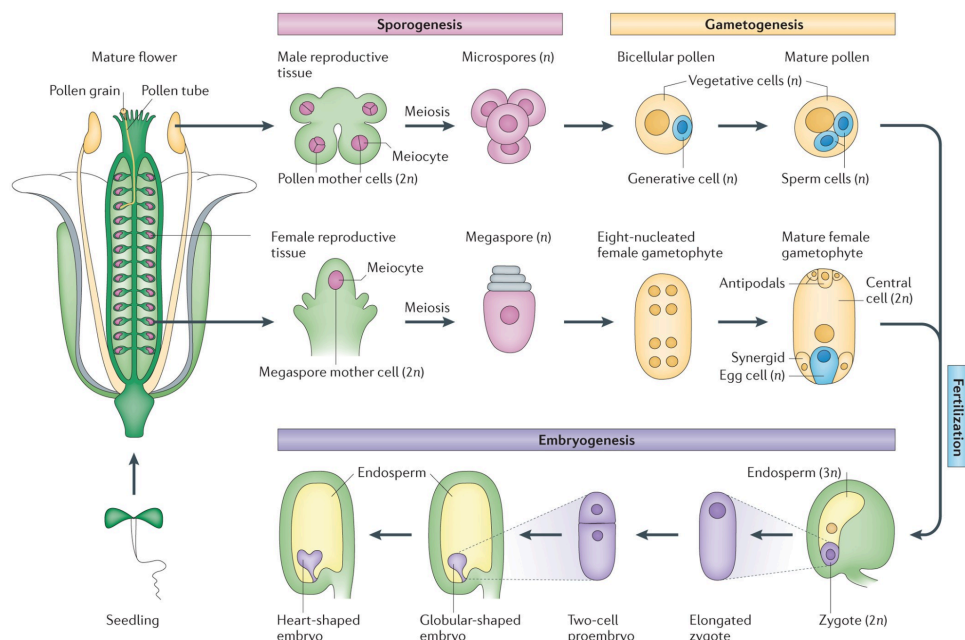


Fig. 1. Schematic representation of the sexual reproduction process in *Arabidopsis thaliana*. Adapted from Kawashima and Berger (2014).

1.2. *Arabidopsis thaliana* and *Oryza sativa*: Similarities and differences

Arabidopsis thaliana and *Oryza sativa* are both well-established plant model species, having been, respectively, the first and second plant genomes to be sequenced (Sasaki and Burr, 2000; The Arabidopsis Genome, 2000).

A. thaliana is a dicotyledonous belonging to the Brassicaceae family. Its flower exhibits the typical structure of eudicots, presenting four types of floral organs organized in concentric whorls (Smyth *et al.*, 1990). Its small size, short life cycle, high seed production, capacity for both cross- and self-pollination, wide ecotype diversity and small genome size have contributed to its establishment as a model organism for angiosperms (Koornneef and Meinke, 2010; Yaschenko *et al.*, 2024). This species can also be transformed by the Floral Dip method, making the generation of mutant lines much faster, easier and cost-effective compared to *in vitro* culture techniques (Clough and Bent, 1998). Currently, numerous genomic tools and resources are available for this species, including a fully sequenced genome, T-DNA insertion mutant libraries and species-specific databases (O'Malley *et al.*, 2015; Yaschenko *et al.*, 2024). As a result, many studies use *A. thaliana* as a proof of concept, initially investigating orthologs of genes of interest in this plant to rapidly infer gene function and narrow down candidate genes before studying them in the target species. However, gene conservation - both in sequence and function - is not always guaranteed, so further functional validation in the target species remains essential.

O. sativa is a monocotyledon belonging to the Poaceae family, and shares significant genetic similarities with other major crops such as wheat, maize and barley (Bennetzen and Ma, 2003; Itoh *et al.*, 2005). This plant is grown in fully inundated crops, making it highly sensitive to water scarcity and drought stress. Rice is predominantly cultivated in Asia, though its cultivation is also expanding across Africa (Jagardish *et al.*, 2015). In Portugal, it is cultivated in the estuarine regions of Tejo, Sado and Mondego rivers (COTArroz, 2023). It's an annual grass with hollow and articulated stems and branches (colms and tillers) that produce panicles

(inflorescences) (Hanada, 1993). Rice's flowers exhibit typical monocot characteristics and are referred to as spikelets. Each spikelet consists of a pair of bract-like sterile organs (lemma and palea) and a single fertile floret containing two lodicules (modified petals), six stamens and one carpel forming the pistil (Guo *et al.*, 2015). Although more complex than *A. thaliana*, *O. sativa* possesses several features that makes it a suitable model for monocot plants, such as a relatively small genome, capacity for both cross- and self-pollination and easy cultivation. Additionally, it's relatively easy to transform using *Agrobacterium tumefaciens*, has a largely sequenced genome and has numerous molecular tools and dedicated databases available (Goff *et al.*, 2002).

1.3. Genetic edition techniques in *O. sativa*

Humans have selected genes and traits in various crops through the process of domestication. However, conventional breeding is time-consuming and presents several limitations, including the unintentional selection of non-target genes and restricted introduction of novel traits due to the inability to cross plants from different species (Tabassum *et al.*, 2021). The rapid growth of the global population, along with ongoing challenges such as climate change has created a pressing need for faster and more precise methods to improve crop performance and meet the rising demand. In response, several gene editing techniques have emerged, allowing exogenous gene insertion into cells and its integration into the genome, and subsequent expression (Khalil, 2020). Beyond their potential in crop improvement, precise genome editing tools also enable functional genetic studies.

Among the various gene editing techniques developed, the most promising ones are the system CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) associated with the Cas9 nickase and other derivative technologies. This method is simple, versatile and highly accurate, enabling the creation of double-strand breaks (DSBs) at specific genomic locations. These breaks are then repaired by the host's cellular machinery through either homology-directed repair (HDR) or non-homologous end joining (NHEJ) (Wang *et al.*, 2017b).

Currently, several variants of the CRISPR system have been developed to overcome limitations associated with the original technique. These include modified versions of the Cas9 protein, such as Cas9 VQR, SpCAS9 (xCas9), and Cas9-NG,

which are capable of recognizing non-canonical PAM sites. This allows genome editing at loci lacking canonical PAM sequences (NGG or CCN) (Hu *et al.*, 2018; Endo *et al.*, 2019; Ren *et al.*, 2019).

Another CRISPR-derived technique is the CRISPR-Cas12a or Cpf1. This system also recognizes non-canonical PAM sequences, specifically T-rich PAMs. Unlike Cas9, Cas12a generates staggered cuts rather than DBSs. An additional advantage is that the Cpf1's coding sequence is smaller than Cas9's sequence, so the designed transformation plasmid is going to be smaller, which in turn improves the transformation efficiency (Alok *et al.*, 2020). This technique has already proven effective for rice transformation, as demonstrated by Malzahn *et al.*, (2019), who successfully edited the *OsROC5* and *OsDEP1* genes while assessing the temperature sensitivity of various Cas12a variants.

Base editing (BE) also utilizes components of the CRISPR system to introduce precise single-base mutations. It uses a catalytically impaired Cas nuclease incapable of creating DBSs, fused to a deaminase enzyme, allowing precise nucleotide conversions. For example, the fusion of this Cas9 with a cytidine deaminase enzyme can convert a cytosine (C) to an uracil (U), resulting in C-T or G-A substitutions (Anzalone *et al.*, 2020). This method was already utilized by Li *et al.*, (2025) to increase the rice tolerance to Bacterial blight (BB) caused by *Xanthomonas oryzae* pathovar *oryzae* (Xoo), by editing the *SWEET14* gene, one of the known susceptibility genes for this disease.

A more recent CRISPR-derived technique involves the use of Cas13, which targets single-stranded RNA (ssRNA) rather than double-stranded DNA (dsDNA). This system is PAM-independent, and preferentially cleaves U-rich regions. As a result, it enables transcriptome manipulation without altering the genome, making its effects reversible. Shuman *et al.*, (2025) demonstrated that this method can be used to knock-down up to six different RNAs simultaneously using distinct crRNAs. In their study, they simultaneously repressed three genes from the same family (*PHOSPHATE STARVATION RESPONSE1* (*OsPHR1*), *OsPHR2*, and *OsPHR3*) which have partially redundant functions. This approach represents a significant advancement in functional genomics, as it allows the suppression of redundant genes in a single transformation, greatly accelerating and reducing the cost of functional studies that previously required the generation of high-level mutants. Furthermore, this study also showed that this method can repress endogenous long noncoding RNAs (lncRNAs). However, the

collateral activity of Cas13, which may lead to nonspecific RNA cleavage, still requires further investigation.

Prime editing enables the precise introduction of various types of mutation into the genome, including base substitutions, small insertions and deletions, etc. This method requires two main components: a reverse transcriptase fused to a Cas9 nickase (PE protein) and a prime editing guide RNA (pegRNA), which contains a spacer sequence complementary to the target DNA and an extension encoding the desired edit. The PE-pegRNA complex binds to one strand of the target DNA and nicks the complementary strand, allowing it to hybridize with the pegRNA. The reverse transcriptase then extends the nicked strand using the pegRNA as a template, incorporating the intended genetic modification. Finally, the host's cellular machinery resolves the resulting heteroduplex by copying the edited strand onto the complementary strand, thereby permanently installing the change (Fig. 2) (Anzalone *et al.*, 2019; Anzalone *et al.*, 2020).

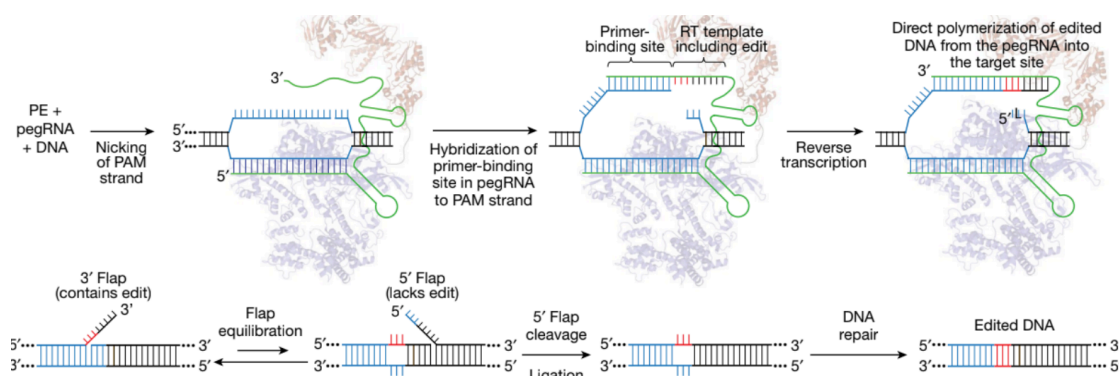


Fig. 2. Schematic representation of the Prime editing technique, which uses a reverse transcriptase fused to a Cas9 nickase (PE) and a prime editing RNA (pegRNA) to introduce precise genome edits. Adapted from Anzalone *et al.*, 2019.

1.4. Impact of the heat stress in *Oryza sativa*

Several abiotic factors, particularly temperature, influence the geographical distribution and seasonal growth of plants (Li *et al.*, 2018; Sharma *et al.*, 2021). However, due to climate change, the global average temperature is rising, with an increase of 0.9°C since the XIX century, and it's projected to rise by an additional 1.5 to

4.5°C during the XXI century (Arora, 2019; Peraudeau *et al.*, 2015). This drastic increase of temperature over a short period of time causes ambient temperatures to exceed the optimal growth temperature of many species, leading to adverse effects due to heat stress (HS) (Quint *et al.*, 2016).

Rice is a staple food for humans, providing approximately 35 to 60% of the daily caloric intake worldwide. Annually, 164 million hectares of rice are cultivated (Fukagawa e Ziska, 2019; Sarma *et al.*, 2023). However, because of the rapid increase of the human population, it is estimated that the production of rice needs to increase by 30% until 2050 to meet global demand (van Dijk *et al.*, 2021). Rice is a versatile crop. cultivated in tropical, subtropical and temperate regions, either in monoculture or in sequential cropping systems (Rao *et al.*, 2017). This species typically grows at temperatures between 27 and 32°C, beyond which it begins to experience adverse effects. The flowering stage requires temperatures of 33°C, with HS occurring at temperatures above 35°C (Kilasi *et al.*, 2018).

Rice is highly sensitive to HS at all developmental stages, and the impact of this stress depends on its intensity, duration and timing. The flowering stage is particularly vulnerable (Cao *et al.*, 2008; Tenorio *et al.*, 2013). As a result, elevated temperatures significantly affect both the quantity and quality of rice grains, influencing traits such as appearance, weight and nutritional value (Jagardish *et al.*, 2021; Tu *et al.*, 2022; Cnossen *et al.*, 2003). For every 1°C increase in temperature, rice yield is estimated to decline by 6.2 to 10%. By 2030, it's expected that 16% of cultivated rice will experience HS for at least five days during the reproductive stage, with projections indicating up to a 41% reduction in productivity by the end of the century (Aryan *et al.*, 2022; Xu *et al.*, 2021; Agnolucci *et al.*, 2020).

The life cycle of rice can be divided in four stages: embryogenesis, vegetative stage, reproductive stage and grain-filling/maturation stage. During embryogenesis, the zygote undergoes successive cell divisions to form a globular embryo. This is followed by the differentiation of the shoot apical meristem (SAM) and root apical meristem (RAM), thereby establishing the plant's primary axis (Itoh *et al.*, 2005). After germination, the vegetative phase begins, during which the plant grows and the SAM produces leaves and tillers as lateral organs (Li, 1979). This is followed by the reproductive phase, marked by a transition in meristem identity from vegetative to floral, leading to the development of flowers. Each flower consists of a lemma, palea, two lodicules, six stamens and a pistil composed of a single carpel. The flowers are

organized in inflorescences called panicles. In this phase, flowering, pollination and fertilization occur. Following embryo formation, it will mature and originate a seed. The seed accumulates storage compounds such as sugars, starch, proteins and other metabolites, which contribute to an increase in seed size and weight. This final stage is referred to as grain filling or maturation (Itoh *et al.*, 2005).

HS has several consequences on the vegetative stage of rice development. Seeds formed in these conditions are smaller, with poorly developed embryos, resulting in reduced viability. Excessive heat also impairs membrane fluidity and thermostability, which decreases germination rates (Cao *et al.*, 2016; Liu *et al.*, 2021). In seedlings, HS disrupts the balance of water, nutrients and phytohormones, leading to stunted growth, leaf yellowing and curling, a decline in photosynthetic rate and, in severe cases, seedling death (Xu *et al.*, 2020; Huang *et al.*, 2019). The roots have a lower optimal growth temperature compared to the aerial parts of the plant, and cease to grow at temperatures above 43°C (González-García *et al.*, 2023).

The reproductive stage is even more sensitive to HS, with a well-documented negative correlation between yield and daily minimum temperature (Arshad *et al.*, 2017). The effects of HS include problems in the panicle initiation and differentiation, a reduction in number and size of spikelets, abnormal floral development, abnormal pollen development, lack of anther dehiscence, low stigmatic receptivity, defects in germination and elongation of the pollen tube and abnormal gametophyte development (Snider *et al.*, 2011; Peraudeau *et al.*, 2015). Heat and drought stress also lead to the accumulation of peroxide in spikelets, causing cellular damage and impairing spikelet fertility (Fu *et al.*, 2015; Shrestha *et al.*, 2022).

HS has a greater impact in anther formation and pollen viability than in ovules, as the latter are located within the female gametophyte and are therefore less exposed to this stress factor (Liu *et al.*, 2021). However, the female reproductive tissues are still affected, although these effects are less studied, HS in female tissues can lead to irregular development of the gametophyte, a reduction in the total number of ovules and an increase in the number of aborted ovules (Lohani *et al.*, 2019).

As in *Arabidopsis*, rice's mature anther is composed of three layers: endothecium, septum and stomium, the latter consisting of a single layer of epidermal cells. Following the first mitotic division of the microspores, starch is deposited within the pollen grains. During anther dehydration, the stomium is weakened by the action of

hydrolytic enzymes. Approximately three hours before anthesis, starch digestion begins, and together with the potassium accumulation, leads to a decrease in osmotic potential and subsequent water uptake, resulting in pollen swelling. Simultaneously, the secondary wall of the endothecium thickens. These changes collectively generate sufficient pressure to rupture the septum and stomium, allowing the release of mature pollen. Under HS, the starch deposition is reduced, leading to premature degeneration of tapetal cells during meiosis, disrupting the microspore nutrition, causing degradation of the pollen wall and resulting in pollen abortion (Arshad *et al.*, 2017; Liu *et al.*, 2020). HS can also reduce carbohydrate metabolism and translocation-related protein activity, leading to a decrease in free sugars, proline and phospholipids in pollen that act as osmolytes. Consequently, water uptake in the locules and pollen grains is reduced, delaying anther wall rupture and causing mislocalization of ubiquitin bodies. This ultimately results in poor anther dehiscence and pollen retention during anther dehiscence (Bonner e Dickinson, 1989; Regman e Yun, 2006; Zhu *et al.*, 2004; Matsui *et al.*, 2000; Sereshen *et al.*, 2014; Peng *et al.*, 2004).

After pollen release, it must reach the stigma, where it will germinate and form the TP. Although it is released in a dehydrated state, rice pollen has a relatively high water content compared to *Arabidopsis*, a thin wall and several microchannels in the exine layer, which facilitate its germination upon contact with the stigma. Upon arrival, water, nutrients and other small molecules are transported from the stigmatic papilla to the pollen grain, enabling its hydration and germination (Doucet *et al.*, 2016). However, these characteristics also make rice pollen highly sensitive to desiccation, rendering it non-viable within five minutes of air exposure due to rapid water loss (Nepi *et al.*, 2001; Pacini e Dolferus, 2019). Under HS, the protein content of the pollen decreases, and the structure and integrity of the cell membranes, composed mainly of lipids and proteins, are adversely affected. HS also reduces the adhesion of pollen to the stigma surface, thereby affecting the pollination success. Collectively, these characteristics significantly reduce pollen viability under these conditions (Das *et al.*, 2014). HS also influences the germination and viability of the TP, by reducing the iron uptake in microspores and pollen tubes (Jagardish *et al.*, 2010).

HS also has a significant negative impact on the quantity and quality of the rice grains produced. One of the most important factors in assessing the grain quality is the amylose content. HS affects rice grain development by shortening the grain-filling period by approximately 3 days for every 1°C increase above the optimal temperature

(25°C) (Tashiro and Wardlaw, 1991). The grain-filling phase involves the redistribution and synthesis of carbohydrates, proteins and lipids within the developing seeds, which are essential for embryo development. HS induces premature leaf senescence, thereby impairing photosynthesis and carbohydrate synthesis (Farooq *et al.*, 2011; Kim *et al.*, 2011). In addition, it affects enzymes involved in starch biosynthesis, such as starch synthase, leading to uneven grain filling, altered amylopectin structure and formation of smaller, irregular starch granules. These changes ultimately reduce the appearance and flavour quality of the grains, which tend to be rounder, loosely packed and often damaged (Ren *et al.*, 2021; Hurkman *et al.*, 2003; Shrestha *et al.*, 2022, Inouchi *et al.*, 2000; Mitsui *et al.*, 2013). The impact of HS on seed production and dormancy cycles also significantly affects seed dispersal, thereby influencing the geographical distribution of the species (Engler *et al.*, 2009).

1.4.1. Sensitive and tolerant rice varieties to HS

Although not sufficient to completely prevent the harmful effects of HS, plants have evolved a range of morphological and physiological strategies to cope with such conditions. Given that different rice cultivars have adapted to specific environments through natural selection and domestication, they have developed distinct mechanisms and varying levels of thermotolerance. In general, *japonica* ecotypes tend to be more sensitive to HS than *indica* cultivars (Riaz *et al.*, 2024). *Nagina22* (N22) cultivar is among the most heat-tolerant varieties, making it a valuable model for studying mechanisms of thermotolerance, allowing us to then use this knowledge to improve the tolerance in economically important rice varieties (Resentini *et al.*, 2023).

Several morphological traits influence the impact of HS during the reproductive stage of rice. Among them, plant architecture plays a particularly important role. When the panicle is surrounded by numerous leaves, the resulting shade and increased local transpiration can cool the panicle, thereby reducing the adverse effects of HS on this structure (Shah *et al.*, 2011). Another relevant factor is stigma position. An enclosed stigma - one that remains inside the spikelet even after anthesis - offers protection against high temperatures and helps prevent the heat-induced decline in fertility. However, stigma exertion - when the stigma protrudes from the spikelet after anthesis - facilitates pollen capture, which can help ensure sufficient pollination even when there's a reduction in anther dehiscence (Chao *et al.*, 2019). The anther traits will also

influence the plant's response to HS. Larger anthers with larger basal and apical pores will release more pollen grains and help compensate for the pollen grains loss caused by the elevated temperature. Additionally, a well-developed anther locule facilitates dehiscence, further enhancing pollen release (Shah *et al.*, 2011).

Rice has a heat signaling pathway that enables it to adjust its physicochemical processes under HS. Heat is perceived by membrane-associated proteins, triggering multiple signaling cascades, including pathways related to intracellular Ca^{2+} levels, production of reactive oxygen species (ROS), heat shock transcription factors (HSFs) and other transcription factors, miRNAs, among others. These components act together to form a complex regulatory network that leads to cellular, physiological and molecular changes aimed at enhancing the plant tolerance to HS (Saini *et al.*, 2022; Baxter *et al.*, 2014).

HSF are among the most critical components regulating transcriptional responses to HS (Scharf *et al.*, 1990). These factors can recognize and bind to heat-shock elements (HSEs) - nGAAnnTTCn or nTTCnnGAAn - thereby activating the expression of genes involved in HS response (Waters, 1995; Tichter *et al.*, 2010; Xiao and Lis, 1988). Lou *et al.*, (2025) analyzed the promoters of all annotated heat-shock proteins (HSP) genes in Arabidopsis, tomato and rice, and identified a conserved HSE consensus motif ATTCTAGAAT. When this sequence was inserted into the promoter of *OsGIF1* (GRAIN INCOMPLETE FILLING 1), coding a cell-wall invertase (CWIN) involved in regulating photoassimilate partitioning during rice grain filling, transgenic plants exhibited improved performance under HS compared to wild type (WT) plants. These results suggest that this HSE motif can be introduced into the promoters of target genes in *Oryza sativa* to drive heat-inducible gene expression, offering a potential strategy for enhancing thermotolerance.

1.4.2. *OsERF98*, *OsBIP2* and *OsYABBY7*

Several genes involved in the HS response have been identified and studied. However, many other intervenient genes with unknown functions remain unknown, particularly the ones specific to the reproductive tissues. González-Schain *et al.* (2016) performed an RNA-seq in pistils from the HS tolerant variety N22, which enabled the identification of differentially expressed genes (DEGs) under HS conditions. The

selection of the genes for this study was based on the log2FC values obtained from the RNA-seq analysis, so the genes with the highest expression increase under HS conditions were selected. Among the most overexpressed genes, those specifically expressed in rice reproductive organs were identified, and selected the ones whose role in the HS response had not yet been characterized. Accordingly, the genes *OsERF98*, *OsBIP2* and *OsYABBY7* were selected.

OsERF98 is a transcription factor predominantly expressed in the anthers and seeds, and is involved in the ethylene-activated signaling pathway. Although the role of this gene in the HS response has not yet been investigated, another member of this family, *OsERF115* has been shown to play a role in both heat and drought tolerance. *OsERF115* enhances the plant's ability to retain water, thereby increasing thermotolerance in rice seeds and plants in the vegetative phase (Park *et al.*, 2021). In Arabidopsis, the ortholog gene *AtERF114* mediates resistance to fungal pathogens and regulates plant architecture, particularly lateral root development, in response to auxin (Li *et al.*, 2022; Canher *et al.*, 2022). However, its role in the HS response remains unknown.

Another gene found to be differentially expressed in HS conditions is *OsBIP2*, an anther-specific HSP associated with TP thermotolerance (Raza *et al.*, 2020). In Arabidopsis, the orthologs *AtBIP1* and *AtBIP2* are linked to female gametogenesis and pollen competitiveness, and the *bip1bip2bip3* triple mutant is lethal to pollen (Maruyama *et al.*, 2015). These genes also appear to be involved in the stress response that, when affected, leads to abnormal pollen development and increased susceptibility of the male gametophyte (Maruyama *et al.*, 2014).

The third gene selected was *OsYABBY7*, expressed in the pistil. Although it has not been well studied in rice, its orthologs in soybean are involved in the response to drought, salinity and ABA stress (Zhao *et al.*, 2017). In cotton, several members of the *YABBY* gene family are downregulated under certain stress conditions (Yang *et al.*, 2018). In Arabidopsis, the ortholog *AtYAB3* is involved with the regulation of the abaxial pattern, lateral organ growth and inflorescence phyllotaxis (Stahle *et al.*, 2009). However, its function in HS conditions remains unstudied.

1.5. Objectives

This study aims to study the *Oryza sativa*'s molecular mechanisms of heat stress tolerance at the reproductive level. With this objective, the genes *OsERF98*, *OsBIP2* and *OsYABBY7* described in 1.4 were selected.

Since analyzing the reproductive phenotypes of multiple rice genes is too time-consuming to yield productive results within the project timeline, *Arabidopsis* was used as a proof of concept to study rice orthologues' phenotypes during anthesis. The model plant *Arabidopsis* can provide fundamental information to understand the role of these genes in HS, and can be easily translated into rice plants (Yaschenko *et al.*, 2024). Thus, the genes *AtERF114* and *AtYAB3*, orthologues of *OsERF98* and *OsYABBY7*, respectively, were selected.

Accordingly to the main objective of this study, the specific aims were:

1. **Validate the RNA-seq data:** To confirm that the selected genes were indeed overexpressed under HS conditions, a RT-qPCR was performed on self-pollinated flowers of the heat-tolerant rice variety N22 under both control and HS conditions. Additionally, the expression of these genes was evaluated in other rice varieties in order to assess their expression patterns across different genetic backgrounds;
2. **Characterize the expression of orthologous genes in *A. thaliana*:** To determine whether the selected orthologous genes exhibit expression patterns similar to those observed in rice, a RT-qPCR was performed on self-pollinated flowers to compare their expression under control and HS conditions;
3. **Generation of reporter and overexpression lines:** To better understand the role of these genes in the HS tolerance mechanism of *Arabidopsis*, constructs for reporter and overexpression lines were generated. The reporter lines will allow the investigation of the expression patterns of these genes in the reproductive tissues, helping to guide the focus of the study. Through phenotypic analysis, the overexpression lines will contribute to elucidate the functional roles of these genes;
4. **Phenotypic analysis of *Arabidopsis* T-DNA insertion mutant lines:** To understand the role of these genes in the HS tolerance mechanism of *Arabidopsis*, a phenotypic analysis of T-DNA insertion mutant lines was

performed, comparing their reproductive success under control and HS conditions;

5. **Generation of constructs for overexpression and Prime Editing lines for *O. sativa*:** Constructs for the development of overexpression and Prime Editing lines targeting the selected genes were generated, with the aim of enabling future creation of rice mutant lines for phenotypic analysis related to the reproductive success under control and HS conditions.

This work aims to provide insights into the CRISPR project by determining whether the selected genes are involved in the HS tolerance mechanism and what their potential roles may be. This study was carried out at SPReDLab in the Faculty of Sciences of the University of Porto (FCUP), and included a 4-month secondment at the University of Missouri (Mizzou) in Professor Bing Yang's laboratory.

2. Material and Methods

2.1. Plant material and growth conditions

Arabidopsis thaliana (L.) wild-type (WT) plants (Columbia-0 ecotype, Col-0) were used, already available at SPReDLab. Seeds of the T-DNA insertion line Salk_086776 (yab3) were obtained from NASC (The European Arabidopsis Stock Center). These plants were sown directly in soil and grown in a controlled growth chamber under long-day conditions (16h light at 21°C / 8h dark at 18°C) with 60% relative humidity.

2.2. Sequence alignments and bioinformatic analysis

The nucleotide sequences from *Arabidopsis thaliana* were obtained using The Arabidopsis Information Resource (TAIR) (<https://www.arabidopsis.org/>) and the NCBI (<https://www.ncbi.nlm.nih.gov/>) databases. The coding sequences of *Oryza sativa* were obtained using the Rice Annotation Project Database (RAP-DB)

(<https://rapdb.dna.affrc.go.jp/index.html>) and the promoter sequences were obtained using PlantPan 4.0 (https://plantpan.itps.ncku.edu.tw/plantpan4/gene_search.php). The bioinformatic analysis of the genes of interest was conducted using the ePLANT RICE Browser tool (https://bar.utoronto.ca/eplant_rice/) for rice, and for Arabidopsis, the Arabidopsis eFP Browser tool (<https://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>) was used.

All DNA sequences obtained from Sanger sequencing experiments were compared with known expected sequences using the APE (A Plasmid Editor) software (<https://jorgensen.biology.utah.edu/wayned/ape/>).

2.3. DNA extraction

The genomic DNA (gDNA) of *A. thaliana* was extracted from young leaves according to Edwards et al. (1991) method.

The gDNA of *Oryza sativa* was extracted from approximately 5 cm long leaf fragments that had been frozen in liquid nitrogen. The leaves were mechanically disrupted using a pestle, followed by the addition of a CTAB extraction buffer (2% CTAB, 20 mM EDTA, 100 mM TrisHCl, 1.4 M NaCl). Samples were incubated in a water bath for 30-40 min. Then, 400µL of chloroform were added, and the solution was centrifuged (14000rpm for 10 min). Then, 400µL isopropanol was added, and after a 10 min incubation, the solution was centrifuged (14000rpm for 10 min) in order to precipitate the DNA. The resulting pellet was washed with 70% ethanol and then resuspended in 50µL of dH₂O.

2.4. Obtention of the *pAtERF114::GUS/YFP*, *pAtYAB3::GUS*, *p35S::AtERF114* and *p35S::AtYAB3* by Gateway Cloning

2.4.1. Bacterial strain and vectors

The bacterial strains used were *Escherichia coli* DH5α and *Agrobacterium tumefaciens* GV3101::PMP90 (resistant to rifampicin and gentamicin).

The plasmids used were pDONR207 (entry vector for the Gateway BP reaction, carrying a gentamicin resistance gene), pB2GW7 (Karimi et al., 2002) (a destination vector containing the constitutive p35S promoter and a spectinomycin resistance gene), pBGWFS7 (Karimi et al., 2002) (a destination vector containing the GUS gene and a spectinomycin resistance gene) and pMT589 (a destination vector containing a 3xNLS:YFP gene and a kanamycin resistance gene). All annotated plasmids are in Supplementary material Figure 32-35.

2.4.2. Growth media and conditions

The *E. coli* cultures were grown in liquid Luria Bertani (LB) medium (1% [w/v] bacto-tryptone, 0.5% [w/v] yeast extract, and 1% [w/v] NaCl) or on solid LB medium (supplemented with 1.5% [w/v] agar). All media were sterilized at 120°C for 20 min in an autoclave. When required, antibiotics (antibiotics and working concentrations used are described in Table 1) were added to the media after sterilization. Solid media were stored at 4°C, while liquid media were stored at room temperature. All *E. coli* cultures were incubated overnight at 37°C, and *A. tumefaciens* cultures were incubated 48h at 28°C. Liquid cultures were incubated with shaking. Bacterial cultures harboring plasmids of interest were preserved in 30% glycerol at -80°C.

Table 1. Antibiotics and respective working concentrations.

Antibiotics	Working concentrations
Gentamicin (Gent)	0.2 µL/mL
Kanamycin (Kan)	1 µL/mL
Spectinomycin (Spec)	1 µL/mL

2.4.3. Plasmids' isolation and purification

The plasmids pDONR207, pB2GW7, pBGWFS7 and pMT589 (Supplementary material Figure 32-35) were extracted from *E. coli* liquid cultures grown from glycerol stocks using the GeneJET Plasmid Miniprep Kit (Thermo Scientific™) according to the

manufacturer's instructions. A restriction enzyme digestion was performed to confirm the identity of the isolated plasmids. The resulting DNA fragments were separated by agarose gel electrophoresis (2.4.4.) and the expected band pattern was confirmed. The restriction enzymes used, and the respective band sizes are listed in Table 2.

Table 2. Restriction enzymes and expected band sizes for the restriction analysis of the empty vectors pDONR207, pB2GW7, pBGWFS7 and pMT589.

Plasmid	Restriction enzymes	Expected bands
pDONR207	Apal + EcoRI	3863 bp 1722 bp
pB2GW7	Apal + EcoRI	9372 bp 1510 bp
pBGWFS7	EcoRV	9219 bp 1711 bp 1290 bp 231 bp
pMT589	Apal	8870 bp 3865 bp

2.4.4. Agarose gel electrophoresis

All PCRs and restriction enzyme's digestions were followed by a electrophoresis on 0.8% [w/v] agarose gel prepared in 1x Sodium Borate (SB) buffer [47g/L boric acid, 200mM NaOH, pH 8.2], with 3µL of GreenSafe Premium (NZYTech) added to the gel before polymerization. The GeneRuler DNA Ladder Mix (Thermo Scientific™) was used as a molecular weight marker, and 1x SB was used as running buffer. The electrophoresis was conducted at 240 V for 7-15 min using an horizontal electrophoresis system (Bio-Rad™). DNA bands were visualized using a UV transilluminator (302-365 nm). Images were captured using a KODAK camera and analysed with the Image Analysis KODAK 1D, version 3.5 (KODAK Scientific Imaging Systems).

2.4.5. DNA amplification and purification

The promoter region and coding sequence (CDS) of AtERF114 and AtYAB3 were amplified by PCR (2.4.6.) using primers with adaptors (attB sites) for the Gateway Cloning system (Supplementary material Table 23). The promoter regions were amplified from the 5'UTR of the upstream gene up to the start codon of the target gene, with a maximum length of 3000 bp. Coding sequences were amplified from the start codon to the stop codon. After the PCR and agarose gel electrophoresis, the promoters and CDS of interest were purified from the gel by using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific™), or directly from the 50µL PCR using the GeneJET PCR Purification Kit (Thermo Fisher Scientific™), depending on the presence or absence of unspecific amplified DNA fragments.

2.4.6. PCR - Polymerase Chain Reaction

The PCR reactions were performed using DreamTaq DNA polymerase (Thermo Scientific™) according to the manufacturer's instructions. The PCR included an initial denaturation step, followed by 34 cycles of denaturation, annealing and extension, with a final extension step and a cooling step. Reactions were carried out in 10µL volumes, as detailed in Table 3. A T100 Thermal Cycler (Bio-Rad™) was used, and the different programs used are listed in Table 4.

Table 3. Components of PCR reactions performed using DreamTaq DNA polymerase (Thermo Scientific™).

Reagents	Volume for a 10 µL reaction
H ₂ O	7.75 µL
10x DreamTaq Buffer (20 mM MgCl ₂)	1 µL
dNTP Mix (10 mM) (Thermo Scientific™)	0.2 µL
Forward Primer (20 µM)	0.25 µL
Reverse Primer (20 µM)	0.25 µL
DreamTaq DNA Polimerase (5 U/µL)	0.05 µL
DNA template	1 µL
Final volume	10 µL

Table 4. PCR conditions using DreamTaq DNA polymerase (Thermo Scientific™).

Step		<i>AtERF114</i> and <i>pAtERF114</i> amplification	<i>AtYAB3</i> and <i>pAtYAB3</i> amplification
Initial denaturation		95°C / 2 min	95°C / 2 min
Denaturation	34 cycles	95°C / 30 s	95°C / 30 s
Annealing		60°C / 30 s	60°C / 30 s
Extension		72°C / 1 min 45 s	72°C / 3 min
Final extension		72°C / 6 min	72°C / 6 min
Cooling		4°C / ∞	4°C / ∞

2.4.7. Gateway Cloning

The Gateway Cloning method (Invitrogen) enables the transfer of DNA sequences between vectors based on the Lambda recombination system. The DNA fragments of interest are flanked by *att* sites and are exchanged between vectors, generating hybrid sequences composed of DNA from both parental vectors. The *ccdB* (*controlling cell death B* gene) gene induces cell death in bacteria transformed with vectors lacking the insert of interest, thereby allowing the selection of the correctly transformed cells. This method involves two recombination reactions - BP and LR - which are illustrated in Figure 3.

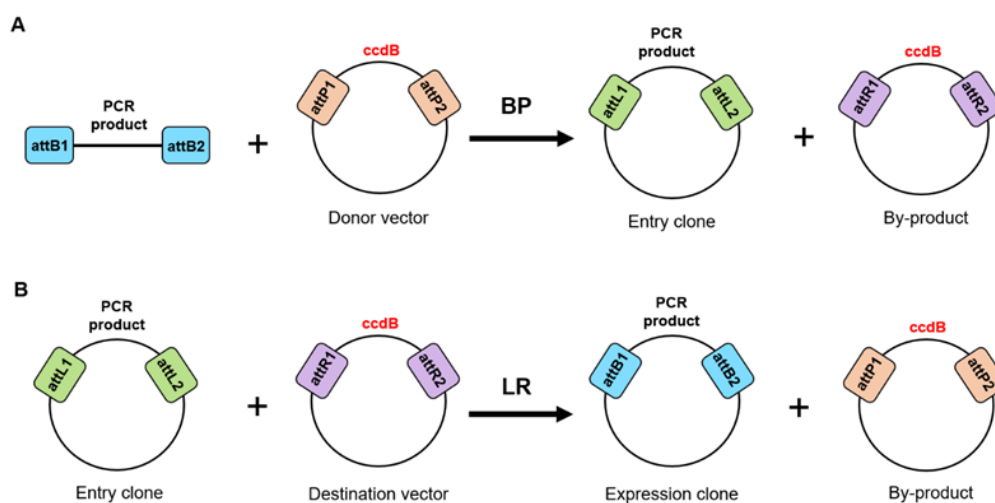


Fig. 3. Schematic representation of the Gateway Cloning System for expression plasmid construction. The BP reaction is catalyzed by the BP Clonase enzyme and facilitates the recombination between the PCR-amplified sequence of interest flanked by *attB* sites and the donor vector sequence flanked by *attP* sites, generating an entry clone containing *attL* sites. The LR reaction is catalyzed by the LR Clonase enzyme, promoting recombination between the *attL*-flanked sequence in the entry construct and the *attR*-flanked sequence in the destination clone, resulting in an expression clone containing *attB* sites. The *ccdB* gene allows the positive selection of colonies containing the desired construct.

In order to introduce the PCR-amplified sequences of interest into the entry vector pDONR207, 3µL of the PCR product and 1µL of the donor vector were mixed, for a total volume of 4µL. Subsequently, 1µL of Gateway™ BP Clonase™ II Enzyme mix (Thermo Fisher Scientific™), was added. The reaction was incubated at 25°C overnight in a thermocycler. On the following day, 1µL of Proteinase K was added to inactivate the reaction, followed by an additional incubation at 37°C for 10 min. One to 1.5 µL of these reactions were used to transform *E. coli* DH5α cells. Positive clones were identified by colony PCR (2.4.9.) and subsequently confirmed by restriction analysis (2.4.11.) and sequencing (2.4.12.).

In order to obtain the expression vectors, 3µL of the entry vector pDONR207 with the desired DNA fragment and 1µL of the respective destination vector were mixed, for a total volume of 4µL, and 1µL of the Gateway™ LR Clonase™ II Enzyme mix (Thermo Fisher Scientific™) was added. The reaction was incubated at 25°C overnight in a thermocycler. On the following day, 1µL of Proteinase K was added to inactivate the reaction, followed by an additional incubation at 37°C for 10 min. One to 1.5 µL of these reactions were used to transform *E. coli* DH5α cells. Positive clones were identified by colony PCR (2.4.9.) and subsequently confirmed by restriction analysis (2.4.11.) and sequencing (2.4.12.).

2.4.8. Preparation of electrocompetent *E. coli* cells

Electrocompetent *E. coli* cells were prepared from liquid LB cultures grown to an optical density at 600 nm (OD₆₀₀) between 0.5 and 0.7. The cultures were divided into 50 mL falcon tubes and centrifuged at 4°C at 4800 rpm for 10 min. The cell pellets were resuspended in 10% glycerol. This centrifugation step was repeated four times. Finally,

the cells were resuspended in 10% glycerol and aliquoted into 50µL portions, frozen in liquid nitrogen, and stored at -80°C.

2.4.9. Bacterial Transformation

The bacterial transformation was performed by electroporation. On ice, 1.5µL of DNA was added to 50µL of electrocompetent cells, and the mixture was transferred to a pre-chilled electroporation cuvette. Following the electroporation pulse in a MicroPulser Electroporator (BioRad), 350µL of LB medium was immediately added to the cells, which were then incubated at 37°C for 45 min without shaking. Finally, the transformed bacteria were plated on LB agar plates containing the appropriate selective antibiotics and incubated at 37°C overnight.

2.4.10. Colony PCR

Colony PCRs were performed as described in section 2.4.6., with the exception that a small portion of the tested colony was used instead of 1µL of DNA template. An initial denaturation step of 5 min was applied to allow bacterial cells lysis and DNA release. This PCR assay was used to identify positive colonies resulting from the respective transformations. The primers used for each reaction were the same used in the initial amplification of the DNA fragment of interest.

2.4.11. Minipreparation of plasmid DNA (Miniprep)

Plasmid DNA extractions for restriction analysis and subcloning procedures were performed using GeneJET Plasmid Miniprep Kit (Thermo Scientific™), following the manufacturer's instructions. For cell harvesting, 3 mL of bacterial culture in LB media was used for all purifications.

2.4.12. Plasmid DNA digestion (Restriction analysis)

The correct orientation of the inserts within the vectors was verified by plasmid DNA digestion, performed according to the manufacturer's instructions (Thermo Fisher™) for the restriction enzymes used. The digestion products were subsequently observed and analysed by agarose gel electrophoresis (2.4.4.). The enzymes used for each construct and the respective expected band sizes are listed in Table 5.

Table 5. Restriction enzymes and expected band sizes for the restriction analysis of the constructs.

Plasmid	Restriction enzymes	Expected bands
<i>pDONR207 + AtERF114 (CDS)</i>	EcoRV + KpnI	3417 bp 1059 bp
<i>pDONR207 + AtYAB3 (CDS)</i>	EcoRV	4067 bp 1589 bp
<i>pDONR207 + pAtERF114</i>	EcoRI + SacI	4025 bp 909 bp
<i>pDONR207 + pAtYAB3</i>	EcoRV	5046 bp 1136 bp
<i>pB2GW7+ AtERF114 (CDS)</i>	Apal + KpnI	9901 bp 432 bp
<i>pB2GW7 + AtYAB3 (CDS)</i>	Apal + EcoRI	11513 bp
<i>pBGWFS7 + pAtERF114</i>	EcoRI + XbaI	10942 bp 1418 bp
<i>pBGWFS7 + pAtYAB3</i>	EcoRI + XbaI	12068 bp 1331 bp 209 bp
<i>pMT589 + pAtERF114</i>	EcoRI + EcoRV	6759 bp 2894 bp 2094 bp 897 bp

2.4.13. Sequencing

The constructs obtained from the BP and LR reactions of the Gateway Cloning system were sequenced to confirm the absence of errors in the sequence of interest. Sequencing was performed by Eurofins Genomics using the Mix2Seq Kit. All sequence data were analysed using the ApE software.

2.4.14. Floral dip

A. thaliana transformation with the constructs obtained was performed according to the floral dip method described by Clough and Bent, (1998). WT plants with a high number of floral buds were used. The siliques and opened flowers were removed prior to transformation to enhance the efficiency of the method. The transformed seeds were collected and sown in soil-based seedbeds, where the transformed plants were selected by spraying with 200 mg/L ammonium glyphosate (BASTA; Bayer Crop Science™) supplemented with 0.1% (v/v) Tween 20. The spraying was performed three to four times, every two days, over a period of 10 days. Resistant plants were subsequently transplanted individually into pots.

2.5. Obtention of the *p35S::OsERF98* e *p35S::OsBIP2* constructs by TEDA cloning

2.5.1. Bacterial strain and vectors

The bacterial strains used were *Escherichia coli* DB3.0. The vector/plasmid used was pBY02 (carrying a kanamycin resistance gene). (Supplementary material Figure 36).

2.5.2. Growth media and conditions

The *E. coli* cultures were grown in the same conditions described in 2.4.2., with the difference that the used media was SOB (Super Optimal Broth) (2% [w/v] bacto-tryptone, 0.5% bacto-yeast extract [w/v], 10 mM NaCl, 2.5 mM KCl) instead of LB.

2.5.3. pBY02 plasmid isolation and purification

The pBY02 plasmid (Supplementary material Figure 36) was obtained by miniprep (2.5.9.). To confirm the identity of the plasmid, a restriction digestion was performed,

and the products were separated by agarose gel electrophoresis (2.5.4.). The restriction enzymes used, and the expected band size are listed in Table 6.

Subsequently, the plasmid was linearized in two steps. First with Sall (New England Biolabs™) and then with Acc65I (Thermo Fisher™), to generate 3'-overhanging cohesive ends. Between the digestion steps, the DNA was purified by ethanol precipitation. For that, the reaction of the first enzyme was inactivated by heat (80°C for 10 min), and 1:10 of NaAc (3M, pH=5.2) was added. Then, the ethanol 100% was added and the solution was incubated at -20°C for 20 min. Then, it was centrifuged (12000 rpm for 15 min) and the pellet was washed two times with ethanol 70%. Finally, the dried pellet was resuspended in 10µL dH₂O.

The fully linearized vector was then purified from an agarose electrophoresis gel (2.5.4.).

Table 6. Restriction enzymes and expected band sizes for pBY02.

Plasmid	Restriction enzymes	Expected bands
pBY02	SacII	8179 bp 4125 bp

2.5.4. Agarose gel electrophoresis

The results of PCRs and restriction analysis were evaluated by electrophoresis on a 1% (w/v) agarose gel prepared in 1x Tris-acetate-EDTA (TAE) buffer. The GeneRuler 1kb DNA Ladder (Thermo fisher™) was used as a molecular weight marker, and the electrophoresis was performed in 1x TAE buffer at 140V for 20-25 min in an electrophoresis tank (17.8 x 25.5 x 6.8cm). A 6x GelRed loading dye was added to each sample to allow DNA visualization. Gel images were acquired using an iBright™ CL750 Imaging System (Thermo fisher™).

2.5.5. DNA amplification and purification

The coding sequences (CDS) of *OsERF98* and *OsBIP2* were amplified by PCR (2.5.6.) using the primers listed in Supplementary material Table 23. The coding sequences were amplified from before the ATG start codon to after the stop codon. The bands of interest were purified from an agarose electrophoresis gel. First the bands were excised and dissolved in sodium iodide (NaI) at 3:1 ratio. Subsequently, silica particles (SiO₂) were added, binding to the DNA and allowing its precipitation by centrifugation (5000 rpm for 30s). The resulting pellet was then washed twice with wash buffer (50% [v/v] ethanol, 10 mM Tris-HCl, pH 7.5-8.0, 1 mM EDTA), dried and resuspended in 10-15µL of H₂O.

2.5.6. PCR - Polymerase Chain Reaction

The PCRs were performed using an in-house Taq DNA polymerase, following the laboratory's standard protocol. Each PCR program consisted of an initial denaturation step, followed by 32 cycles of denaturation, annealing and extension, and concluded with a final extension step and a cooling phase. Reaction mixtures of 20µL were used (Table 7). A C1000 Touch Thermal Cycler (Bio-Rad™) was used, and the programs are listed in Table 8.

Table 7. Components of PCR reactions performed using an in-house Taq DNA Polymerase.

Reagents	Volume for a 10 µL reaction
H ₂ O	12 µL
10x Buffer	2 µL
dNTP Mix (10 mM)	1.5 µL
Forward Primer (10 µM)	1 µL
Reverse Primer (10 µM)	1 µL
DMSO	1 µL
DNA Polimerase (5 U/µL)	0.5 µL
DNA template	1 µL
Final volume	20 µL

Table 8. PCR conditions using an in-house Taq DNA Polymerase.

Step		<i>OsERF98</i> amplification	<i>OsBIP2</i> amplification
Initial denaturation		95°C / 3 min	95°C / 3 min
Denaturation	32 cycles	95°C / 30 s	95°C / 30 s
Annealing		53°C / 30 s	52°C / 30 s
Extension		72°C / 1 min	72°C / 2 min 45 s
Final extension		72°C / 6 min	72°C / 6 min
Cooling		12°C / ∞	12°C / ∞

2.5.7. TEDA cloning

The TEDA (T5 exonuclease DNA assembly) cloning method is a technique derived from Gibson assembly and the Hot Fusion method, which enables the directional cloning of DNA fragments using only T5 exonuclease. This method uses a pre-linearized vector designed to generate 3'-overhangs at the insertion site. The DNA fragments of interest are amplified by PCR using primers containing 20-40 nucleotides of overlap with regions adjacent to the intended insertion site. During the reaction, the DNA fragments anneal to the target vector, forming a circular DNA molecule with nicks. Unlike Gibson assembly, in TEDA cloning these nicks in the plasmid are not repaired *in vitro* but instead they are repaired inside *E. coli* following transformation (Fig. 4) (Xia *et al.*, 2019). Reaction mixtures of 20µL were used (Table 9). The assembly reaction was incubated in an C1000 Touch Thermal Cycler (Bio-Rad™) at 30°C for 1 hour.

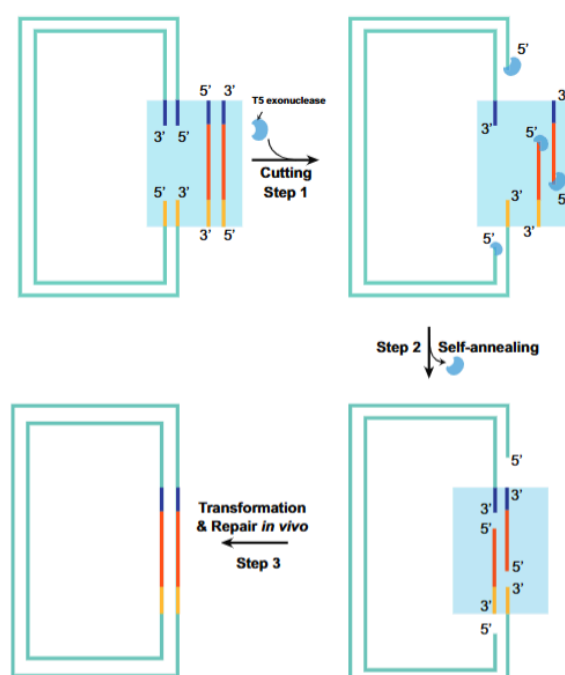


Fig. 4. Schematic representation of the TEDA cloning method. The blue rectangle represents the linearized plasmid, while the red segment indicates the DNA insert. Ends with the same color denote regions of homology. T5 exonuclease digests the 5' ends of both vector and the insert, generating compatible overhangs that allow annealing between the plasmid and the insert sequences. The resulting construct is used to transform *E. coli*, where the nicks between the insert and vector are repaired. Adapted from Xia *et al.*, (2019).

Table 9. Components for the TEDA cloning reaction.

Reagents	Volume for a 20 μ L reaction
DNA insert (~100ng/ μ L)	3:1
Purified linearized vector (~100ng/ μ L)	100-200 ng
5x TEDA	4 μ L
H ₂ O	to 20 μ L
Total	20 μ L

2.5.8. Bacteria Transformation

The *E. coli* transformation was performed via heat shock. 5-7 μ L of construct DNA were added to 60 μ L of chemically competent DB3.0 cells, and the mixture was incubated on ice for 20 min. The cells were then subjected to heat shock at 42°C for 50 seconds, and immediately returned to ice. Then, 500 μ L of SOB (Super Optimal Broth) medium was

added. The cultures were incubated at 37°C for 1 hour with shaking, and then plated onto LB agar plates containing the appropriate selective antibiotic and incubated overnight at 37°C.

2.5.9. Colony PCR

Colony PCRs were performed as described in section 2.5.6., with the exception that a small portion of the tested colony was used instead of 1µL of DNA template. An initial denaturation step of 5 min was applied. This PCR assay was used to identify positive colonies resulting from the respective transformations. The primers used for each reaction were the same used in the initial amplification of the sequence of interest.

2.5.10. Minipreparation of plasmid DNA (Miniprep)

Plasmid DNA extractions for restriction analysis and subcloning procedures were performed from 2mL of liquid bacterial culture grown in LB medium. After centrifugation, the bacterial pellet was resuspended in 300µL of P1 solution (Table 10). Subsequently, 300µL of P2 solution (Table 10) were added to lyse the cells, followed by the addition of 300µL of P3 solution (Table 10) to neutralize the reaction. The samples were centrifugated, and the supernatant was transferred to a new tube, where 650µL of isopropanol were added to precipitate the DNA. The resulting pellet was washed twice with 500µL of 70% ethanol and finally resuspended in 50µL of H₂O.

Table 10. Composition of the P1, P2 and P3 solutions used in minipreparation of plasmid DNA.

P1	P2	P3
50 mM Tris base.....6.06 g	200 mM NaOH.....8g	H ₂ O.....500 mL
10mM Na ₂ EDTA.2H ₂ O.....3.72 g	1% SDS.....10g	Potassium acetate.....294.5 g
HCl.....to pH8	H ₂ O.....to 1000 mL	Glacial acetic acid.....110 mL
H ₂ O..... to 1000mL		H ₂ O.....to 1000 mL

2.5.11. Restriction analysis

The correct orientation of the inserts within the vectors was verified by plasmid DNA digestion, performed according to the manufacturer's instructions for the restriction enzymes used. The digestion products were subsequently observed and analysed by agarose gel electrophoresis (2.5.4.). The enzymes used for each construct and the respective expected band sizes are listed in Table 11.

Table 11. Restriction enzymes and expected band sizes for the restriction analysis of the constructs.

Plasmid	Restriction enzymes	Expected bands
<i>pBY02 + OsERF98 (CDS)</i>	AatII	9778 bp 2286 bp 1164 bp
<i>pBY02 + OsBIP2 (CDS)</i>	SacI	12785 bp 1081 bp 852 bp 132 bp

2.5.12. Sequencing

The constructs obtained were sequenced to confirm the absence of errors in the sequence of interest. All sequence data were analysed using the ApE software.

2.6. Obtention of the Prime Editing construct for introduction of an heat-activated switch in the *OsYABBY7* gene promoter

2.6.1. Bacterial strain and vectors

The bacterial strains used were *Escherichia coli* DB3.0. The vector/plasmid used was pAG731 (carrying a kanamycin resistance gene) (Supplementary material Figure 37). This plasmid also contains a GFP (Green Fluorescence Protein) expression gene that serves as a marker for distinguishing colonies transformed with vectors containing or

lacking the insert of interest. During the Golden Gate assembly reaction, this gene is removed and replaced by the insert. As a result, colonies harboring the insert will not exhibit fluorescence, while colonies transformed with the empty plasmid will display green fluorescence.

2.6.2. Growth media and conditions

The growth media and conditions used were the same as those described in 2.5.2.

2.6.3. Obtention of the pBY02 plasmid

The pAG731 plasmid (Supplementary material Figure 37) was obtained by miniprep (2.5.9.). To confirm the identity of the plasmid, a restriction was performed, and the products were analysed by agarose gel electrophoresis (2.5.4.). The restriction enzymes used and the expected band sizes are listed in Table 12.

Table 12. Restriction enzymes and expected band sizes for pAG731.

Plasmid	Restriction enzymes	Expected bands
pAG731	Acc65I	12859 bp 5689 bp

2.6.4. Prime Editing vector design

2.6.4.1. *OsYABBY7* promoter analysis

Promoter analysis and selection of the insertion site were carried out as described by Lou *et al.*, (2025). The promoter was first analysed for active cis-elements, as the insertion could not disrupt other regulatory elements of the gene. This analysis was performed using the PlantCARE database (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). Since the efficiency of proper regulation of the target gene decreases with the increase of the distance from

the insertion site, a maximum distance of 1 kb from the start codon (ATG) was established.

2.6.4.2. Obtention of the *spacer* and *extension* sequences

pegRNA design was carried out as described by Gupta *et al.*, (2024). The pegRNA consists of two main elements: a spacer, which is complementary to the target strand, and an extension, which serves as a template for reverse transcription. The extension itself is composed of three elements: the template, which contains the desired edit and acts as the reverse transcriptase template; the PBS (Primer Binding Site), which anneals to the target strand; and a linker, a short sequence of approximately 8 bp that connects the reverse transcriptase to Cas9.

The protospacer adjacent motifs (PAMs), either NGG or CCN, closest to the intended insertion site were identified, and the spacer and extension sequences were designed using PlantPegDesigner (<http://www.plantgenomeediting.net/>). The linker connecting the PBS and the pseudoknot was designed using pegLIT (<https://peglit.liugroup.us>). The final primers are listed in Supplementary material Table 23.

Primers were then annealed in a thermocycler using the program described in Table 14 to generate a double-stranded DNA (dsDNA) molecule with 3' overhangs compatible with the destination vector pAG731.

Table 13. Components for the primer annealing reaction for the spacer (*pegYABBY7*) and extension (*extYABBY7*).

Reagents	Volume (µL)
10x PNK (Polynucleotide Kinase)	2 µL
Forward Primer (10 µM)	2 µL
Reverse Primer (10 µM)	2 µL
T4 PNK	0.2 µL
H ₂ O	13.8 µL
Total	20 µL

Table 14. Thermocycler program used for the primer annealing reaction for the spacer (*pegYABBY7*) and extension (*extYABBY7*).

Temperature (°C)	Time (min)
37°C	60 min
95°C	10 min
85°C	1 min
75°C	1 min
65°C	1 min
55°C	1 min
45°C	1 min
35°C	1 min
25°C	1 min
12°C	∞

2.6.4.3. Golden Gate Assembly

The Golden Gate Assembly technique enables the assembly of up to nine DNA fragments into a destination vector in a defined order and within a single reaction. This method relies on the ability of type IIS restriction enzymes to cut outside their recognition sites, allowing two DNA fragments flanked by compatible restriction sites to be digested and ligated. After ligation, the recognition site is no longer present in the final construct, preventing further digestion. However, if the empty vector re-ligates, the recognition site is restored and the vector is re-digested, allowing another opportunity for correct assembly. In this way, the fragments of interest, destination vector, restriction enzyme and ligase are combined in a single tube and incubated in a thermocycler (Fig. 5) (Engler e Marillonnet, 2014).

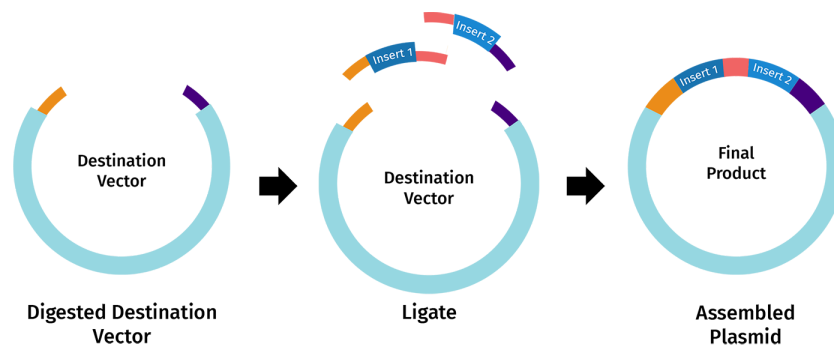


Fig. 5. Schematic representation of the Golden Gate Assembly technique. This method uses type IIS restriction enzymes to ligate multiple fragments in the desired order into a destination vector within a single reaction. Matching colors indicate compatible ends. Adapted from SnapGene Golden Gate Assembly Guide.

To introduce the pre-incubated and annealed sequences (2.6.4.2.) into the destination vector pAG731 (Supplementary figure 37), a single reaction containing both the restriction enzyme and DNA ligase was performed, as described in Table 15. The reaction was incubated in a thermocycler using a program consisting of 50 cycles of restriction (37°C for 30 min) and ligation (16°C for 10 min) (Table 16).

Table 15. Components for the Golden Gate Assembly reaction.

Reagents	Volume (µL)
<i>pegOsYABBY7</i>	2 µL
<i>extOsYABBY7</i>	2 µL
<i>pAG731</i>	2 µL
10x T4 buffer	1.5 µL
ATP	1 µL
T4 ligase	1 µL
BsaI-HF	1 µL
H ₂ O	4.5 µL
Total	15 µL

Table 16. Thermocycler program used for the Golden Gate Assembly reaction.

Step		Temperature	Time
Restriction	50 Cycles	37°C	30 min

Step		Temperature	Time
Ligation		16°C	10 min
Restriction		37°C	10 min
Final restriction		37°C	30 min
Deactivation		80°C	10 min
		12°C	∞

2.6.5. Bacteria Transformation

E. coli transformation was performed as described in 2.5.8. The verification of the transformation was based on GFP expression, since the coding sequence for this protein is removed in constructs containing the insert of interest. Thus, colonies exhibiting fluorescence were considered negative for the insertion, while non-fluorescent colonies were considered positive. A colony PCR was subsequently performed on the non-fluorescent colonies to further confirm the presence of the insert.

2.6.6. Miniprep of plasmid DNA (Miniprep)

The miniprep of the plasmid DNA was performed as described in 2.5.10.

2.6.7. Plasmid DNA digestion (Restriction analysis)

The restriction analysis to confirm the correct orientation of the inserts was performed as described in 2.5.11. The enzyme used for this construct was PfiMI. However, because the plasmid map was outdated, the expected band sizes did not correspond, and the confirmation was made based on the differences observed in the empty plasmid band pattern.

Table 17. Restriction enzymes for the restriction analysis of the constructs.

Plasmid	Restriction enzymes
pAG731 + peg/extOsYABBY7	PfiMI

2.6.8. Sequencing

The sequencing of the resulting construct was performed as described in 2.5.12.

2.7. Gene expression analysis

2.7.1. RNA extraction and quantification

RNA from WT and *yab3* mutant *A. thaliana* plants was extracted from approximately 10 mg of inflorescence tissue using PureZOL™ RNA Isolation Reagent, following the manufacturer's instructions (Bio-Rad™). RNA samples of the rice varieties N22, Nipponbare (Nipp) and Kitake (Kit) from plants grown in normal and HS conditions were kindly provided by Dr. Nelson Saibo and Dr. Tiago Santos from ITQB NOVA (Lisbon, Portugal).

2.7.2. cDNA synthesis

Following RNA extraction, 0.5µL of RiboLock RNase Inhibitor was added to each sample, and reverse transcription was performed. The resulting cDNA was tested using primers for the housekeeping gene *AtACTIN8* (*AtACT8*) for Arabidopsis samples, and primers for *ETHYLENE RESPONSE FACTOR 98* (*OsERF98*) for *O. sativa*, to confirm the absence of genomic DNA contamination (Table 18). The same protocol was used for previously extracted RNA samples of the rice varieties N22, Nipponbare (Nipp) and Kitake (Kit) from plants grown in normal and HS conditions.

Table 18. PCR conditions using DreamTaq DNA Polymerase.

Step		<i>AtACT8</i> amplification
Initial denaturation		95°C / 2 min
Denaturation	34 cycles	95°C / 30 s
Annealing		58°C / 30 s
Extension		72°C / 30 s

Final extension		72°C / 5 min
Cooling		12°C / ∞

2.7.3. Real-Time quantitative polymerase chain reaction (RT-qPCR)

To determine whether the *yab3* line represented a knockout or knockdown for the gene under study, and to compare its expression levels in WT and *yab3* plants under normal and HS conditions (2.8.3.), RT-qPCR was performed using the primers listed in Supplementary material Table 23. This technique was also used to compare the expression levels of *OsERF98*, *OsBIP2* and *OsYABBY7* in rice varieties N22, Nipp and Kit under both control and HS conditions. For both *A. thaliana* and *O. sativa*, primers were designed using Primer3Plus (<https://www.primer3plus.com/index.html>). The housekeeping genes *ACTIN8* (*AtACT8*) and *RELATED DO UBIQUITIN1* (*AtRUB1*) were used as reference genes for *A. thaliana*, while *NUCLEIC ACID BINDING PROTEIN* (*OsNABP*) and *EARLINESS 1* (*OsEF1*) were used for *Oryza sativa*. All primer sets were tested for efficiency and specificity using serial dilutions of cDNA, allowing the generation of calibration curves used to quantify plant cDNA in each sample. Amplification was performed using SSoFast™ SYBR® Green Supermix (Bio-Rad) with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). For each experimental condition, three technical replicates were carried out. The thermocycler program is described in Table 19. Following amplification, a dissociation curve analysis was performed by increasing the temperature from 60 to 95°C to confirm specificity. Data was analysed using CFX Maestro™ Software (Bio-Rad).

Table 19. RT-qPCR conditions using SSoFast™ SYBR® Green Supermix (Bio-Rad).

Step		
		95°C / 3 min
Denaturation		95°C / 10 s
Denaturation	34 cycles	95°C / 10 s
Annealing		60°C / 30 s

2.8. Reproductive phenotype analysis of the *yab3* mutant line

2.8.1. Genotyping

The *yab3* mutant plants were genotyped by PCR (2.4.6.) (Table 20) to determine whether they were homozygous (HM) for the T-DNA insertion. Three primers were used to detect the presence/absence of the insertion. The LP (left primer) and RP (Right primer) amplify the WT band in the absence of a T-DNA insertion. If the insertion is present, these primers are too far apart to generate a product, and no WT band is formed. The BP (border primer) is specific to an end of the T-DNA insertion. In the presence of the insertion, amplification occurs between BP and LR, generating the mutant (mt) band. The PCR products were visualized and analysed by agarose gel electrophoresis (2.4.4.) to assess the banding patterns and identify homozygous mutants (MT), heterozygous (HT) and WT plants. The WT fragment is always larger than the MT fragment, as the genomic sequence between LP and RP is longer than that between BP and either LP or RP. The primers used are listed in Supplementary material Table 23.

Table 20. PCR conditions using DreamTaq DNA Polymerase.

Step		<i>yab3</i> genotyping
Initial denaturation		95°C / 2 min
Denaturation	34 cycles	95°C / 30 s
Annealing		52°C / 30 s
Extension		72°C / 1 min 12 s
Final extension		72°C / 6 min
Cooling		12°C / ∞

2.8.2. Heat stress assay during anthesis

WT and *yab3 A. thaliana* plants were subjected to a HS assay to study the behaviour of genes of interest under this stress condition. Reproductive mature plants previously grown under optimal conditions (2.1.) were exposed to 38°C for 4 hours (10 AM - 2 PM)

at 60% relative humidity, in a single treatment. Prior to the HS period, the temperature was gradually increased to 38°C, replicating natural conditions, and similarly decreased back to optimal conditions afterward. Prior to the assay, all plants were prepared by removing all siliques and open flowers that might have already been pollinated, ensuring that only flowers at anthesis were subjected to HS.

Flowers exposed to HS during anthesis were collected after a 24h recovery period for RNA extraction and aniline blue staining. Siliques derived from heat-stressed flowers were marked and allowed to develop fully before being harvested, cleared and photographed for phenotypic analysis.

2.8.3. Histochemical detection of GUS

Inflorescences from the *pAtYAB3::GUS* reporter line were collected and fixed overnight at 4°C in a 1:9 acetic acid:ethanol solution. Samples were then washed for 10 min in a 90% ethanol followed by 10 min in 70% ethanol. Subsequently, they were incubated overnight at 4°C in a clearing solution consisting of 160g of chloral hydrate (Sigma-Aldrich) dissolved in 100mL of water and 50mL of glycerol. Samples were observed using a Zeiss Axiolmager AZ microscope equipped with differential interference contrast (DIC), and images were captured with a Zeiss Axiocam MRc3 camera with the Zen Imaging (Zen 2011 SP1) software.

2.8.4. Pollen tube staining with decolorized aniline blue

Self-pollinated *A. thaliana* pistils from WT and *yab3* plants were fixed overnight in a 1:9 acetic acid:ethanol solution, followed by three 5 min washes in distilled water. Samples were then cleared by incubation in 1M NaOH overnight, followed by three 20 min washes in water. The material was subsequently incubated in 0.1% (w/v) aniline blue solution at 4°C. Samples were observed using a Leica DMLB epifluorescence microscope. Images were captured with a DS-Ri2 camera (Nikon) in automatic exposure mode and processed with Nikon NIS-Elements software.

2.8.5. Clearing of siliques

The 10 oldest siliques from each main stem were collected, excluding the first two at the base, from plants grown under control (C) conditions. For the HS conditions, the flowers subjected to HS were marked and then fully developed under optimal conditions, and the siliques originated from them were collected. Siliques were collected from both WT and *yab3* plants. All samples were fixed overnight at 4°C in a 1:9 acetic acid:ethanol solution, then washed in 90% ethanol for 10 min. Finally, siliques were transferred to 70% ethanol and stored at 4°C until observation and imaging under a stereomicroscope.

2.9. Statistical Analysis

The data originated from RT-qPCR and phenotypic analysis was analysed statistically using GraphPad Prism 8 software (www.graphpad.com). The expression levels of the target genes and the number of fertilized ovules were compared between WT and mutant plants, and between normal and HS conditions. A two-way ANOVA and t-test statistical tests were used to analyse the gene expression results, while only a two-way ANOVA statistical test was used to analyse phenotypic differences.

3. Results

3.1. Candidate genes in *A. thaliana* and *O. sativa*

3.1.1. *O. sativa* candidate genes' analysis

To identify the candidate genes for this study, the list of the overexpressed genes in pollinated pistils from the heat-tolerant rice variety N22, under HS conditions, obtained from the RNA-seq dataset reported by González-Schain *et al.* (2016), was analyzed.

A literature review was conducted for each gene to gather information on the tissues where they are expressed, subcellular localization, expected molecular function, and previously described mutant phenotypes. (Supplementary Table 22).

From this list, priority was given to genes that showed higher levels of overexpression under HS conditions and were specifically expressed in reproductive tissues, as this developmental stage was the focus of our study. Based on these criteria, *OsERF98*, *OsBIP2*, and *OsYABBY7* were selected for further analysis.

Orthologous *Arabidopsis thaliana* genes corresponding to the candidate genes in *O. sativa* were identified (Table 21), and *AtERF114* and *AtYAB3* were selected for further analysis in *A. thaliana*.

Table 21. Candidate genes in *O. sativa* and respective orthologues in *A. thaliana*. The genes selected for this study are colored in blue.

<i>O. sativa</i>	<i>A. thaliana</i>
<i>OsERF98</i>	<i>AtERF114</i>
	<i>AtRap2.6L</i>
<i>OsBIP2</i>	<i>AtBIP1</i>
	<i>AtBIP2</i>
	<i>AtBIP3</i>
<i>OsYABBY7</i>	<i>AtYAB3</i>

3.1.2. Gene expression levels in *O. sativa* inflorescences in control vs. HS conditions

RT-qPCR was performed to investigate the expression patterns of *OsERF114*, *OsBIP2* and *OsYABBY7* in pollinated rice flowers from three cultivars: N22 (a heat-tolerant variety), Nipponbare (Nipp) and Kittake (Kit), under control (N) and HS (38°C) conditions (Fig. 6).

Prior to expression analysis, efficiency for *OsYABBY7* primers was evaluated and found to be within the acceptable range of 80-120%, with correlation coefficients (R^2) exceeding 0.0990, confirming the linearity of the calibration curve derived from serial dilutions of cDNA (Supplementary material Figure 38). Melt curve analysis revealed a

single peak for this gene, indicating the absence of non-specific amplification (Supplementary material Figure 38 B). The primers for *OsERF98* and *OsBIP2* did not meet these quality parameters and are currently under further optimization. As such, expression data for these genes could not be obtained at this stage.

Expression analysis of *OsYABBY7* revealed a significant upregulation under heat stress conditions across all three varieties when compared to the control (Fig. 6) ($p = 0.0003$ in Nipp; $p < 0.0001$ in N22; $p = 0.0005$ in Kit), with the highest increase observed in the heat-tolerant cultivar N22.

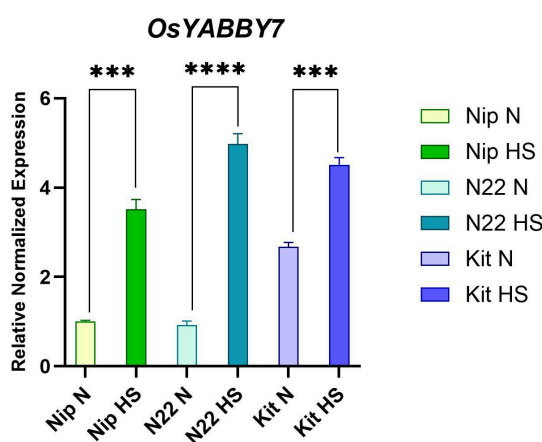


Fig. 6. Relative expression of *OsYABBY7* in control (N) and HS conditions in three different rice varieties: Nipp, N22 and Kit. In all varieties, a significant increase in gene expression was revealed under HS conditions, with the highest increase observed in the heat-tolerant variety N22. The expression levels were normalized according to the reference genes *OsNABP* and *OsEF1*. Each bar represents the mean of three biological replicates. Asterisks indicate statistically significant differences according to Sidak test ($p \leq 0.05$).

3.1.3. Gene expression levels in *A. thaliana* flowers in control vs. HS conditions

RT-qPCR was conducted to evaluate the expression of the *A. thaliana* orthologs of the candidate rice genes under HS conditions. The expression of *AtERF114* and *AtBIP2* was assessed in pollinated Arabidopsis flowers subjected to control (N) and HS (38°C) conditions (Fig. 7).

The primer pairs used for both genes were evaluated and found to be within the acceptable range of 80-120%, with correlation coefficients (R^2) exceeding 0.0990,

confirming the linearity of the calibration curve derived from serial dilutions of cDNA (Supplementary material Figure 39 and 40). Melt curve analysis revealed a single peak for this gene, indicating the absence of non-specific amplification (Supplementary material Figure 39 B, Figure 40 B).

For *AtERF114*, a significant downregulation was observed under HS compared to control conditions ($p = 0.0034$).

For *AtBIP2*, a slight increase in expression was detected under HS. However, this change was not statistically significant ($p = 0.1257$).

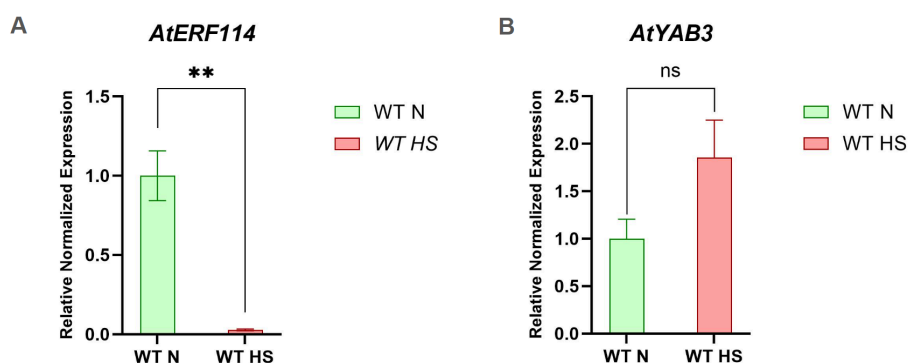


Fig. 7. Relative expression of *AtERF114* and *AtYAB3* in control (N) and HS conditions in *A. thaliana*. A - a significant decrease in *AtERF114* expression was revealed under HS conditions. B - Non-significant increase in the *AtBIP2* expression in HS conditions. The expression levels were normalized according to the reference genes *AtACT8* and *AtRUB1*. Each bar represents the mean of three biological replicates. Asterisks indicate statistically significant differences according to Sidak test ($p \leq 0.05$).

3.2. *In silico* analysis of the candidate genes in *A. thaliana* and *O. sativa*

3.2.1. *In silico* analysis of *OsERF98*, *OsBIP2* and *OsYABBY7*

A bioinformatic analysis was conducted for the *O. sativa* genes *OsERF98* (LOC_Os02g34260), *OsBIP2* (LOC_Os03g50250), and *OsYABBY7* (LOC_Os07g38410) using the ePlant Rice Browser tool

(https://bar.utoronto.ca/eplant_rice/). This platform enabled the investigation of gene expression patterns across different tissues. The expression data provided by the eFP Browser are derived from published microarray and RNA-seq datasets.

OsERF98 is predominantly expressed in the seed, particularly in the embryo. Its expression increases throughout seed development, reaching a peak at stage S5. This gene is also expressed in anthers, and its expression is considerably lower in female reproductive tissues (Fig. 8).

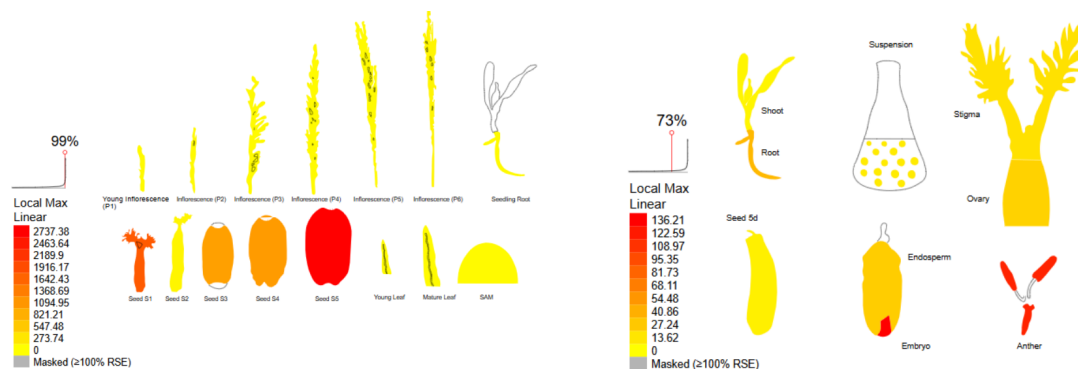


Fig. 8. Predicted expression pattern of *OsERF98* in the reproductive tissues of *O. sativa*, obtained from the Rice eFP browser database.

OsBIP2 is also predominantly expressed in seeds. However, in contrast to *OsERF98*, it shows higher expression during the early stages of seed development, and is more highly expressed in the endosperm than in the embryo. It is expressed in both female and male reproductive tissues, exhibiting similar expression levels in the stigma and anthers (Fig. 9).

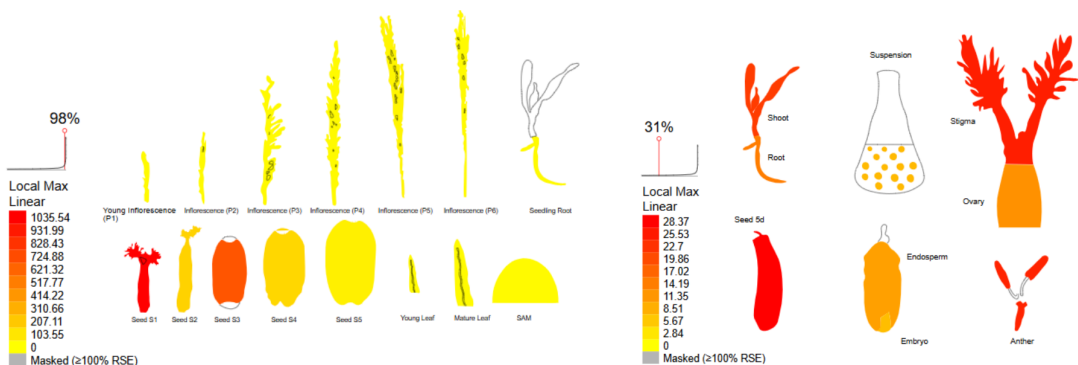


Fig. 9. Predicted expression pattern of *OsBIP2* in the reproductive tissues of *O. sativa*, obtained from the Rice eFP browser database.

Finally, *OsYABBY7* is expressed throughout the reproductive phase of *O. sativa*, especially during the early stages of inflorescence development and the late stages of seed development, particularly in the endosperm. Its expression is higher in female reproductive tissues, especially in the ovary (Fig. 10).

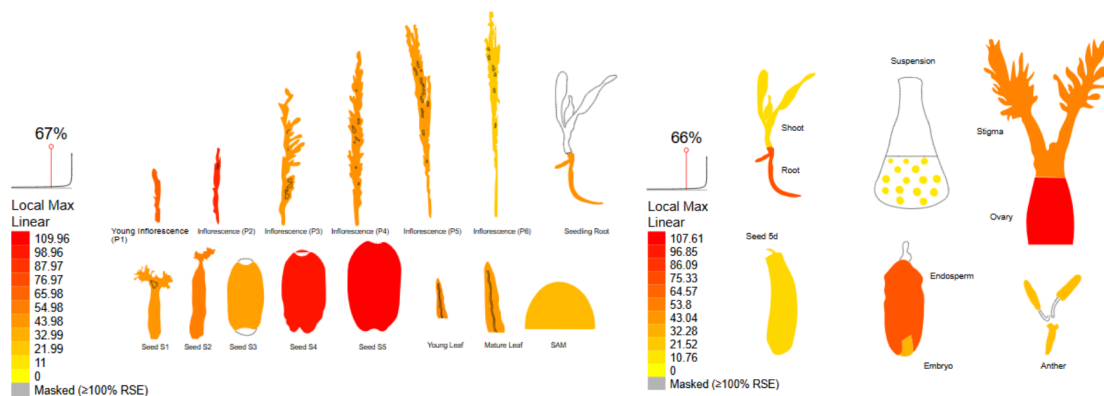


Fig. 10. Predicted expression pattern of *OsYABBY7* in the reproductive tissues of *O. sativa*, obtained from the Rice eFP browser database.

3.2.2. *In silico* analysis of *AtERF114* and *AtYAB3*

A bioinformatic analysis was conducted for the *A. thaliana* genes *AtERF114* (AT5G61890.1) and *AtYAB3* (AT4G00180) using the Arabidopsis eFP Browser tool (<https://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>). This platform enabled the investigation of gene expression patterns across various reproductive tissues and developmental stages. The expression data provided by the eFP Browser are derived from published microarray and RNA-seq datasets.

AtERF114 (Fig. 11) is predominantly expressed in female reproductive tissues, with relatively uniform expression levels, except in the stigma, where expression is slightly reduced. In male tissues, *AtERF114* shows lower expression levels, which remain relatively constant throughout pollen development. Following pollen release, the expression of this gene drops and remains low up to four hours, becoming nearly undetectable in elongated pollen tubes.

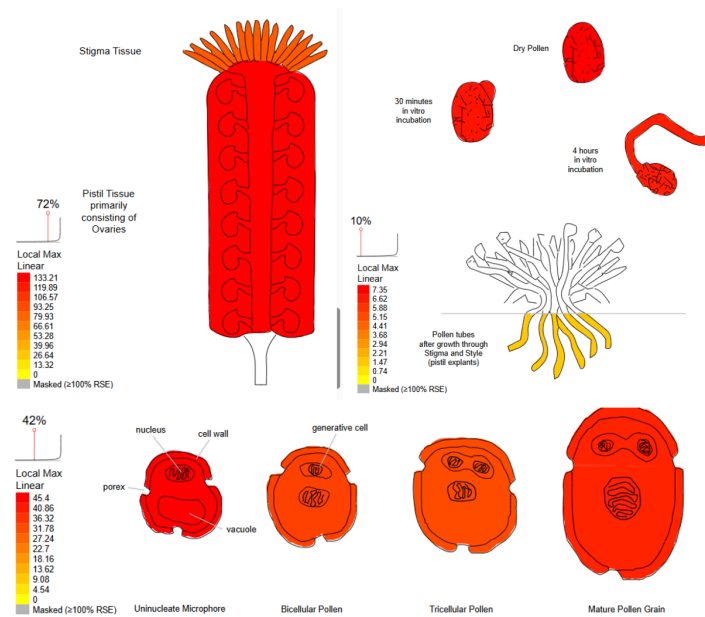


Fig. 11. Predicted expression pattern of *AtERF114* in the female reproductive tissues, pollen development and pollen grain during *in vitro* incubation, germination and PT growing, obtained from the Arabidopsis eFP browser database.

AtYAB3 (Fig. 12) also exhibits higher expression levels in female reproductive tissues compared to male tissues, although, similarly to *AtERF114*, its expression is lower in the stigma than in the other female organs. In male tissues, *AtYAB3* expression increases progressively during pollen grain development, declining at the mature pollen stage. Following pollen release, its expression remains very low during both pollen grain incubation and pollen tube growth.

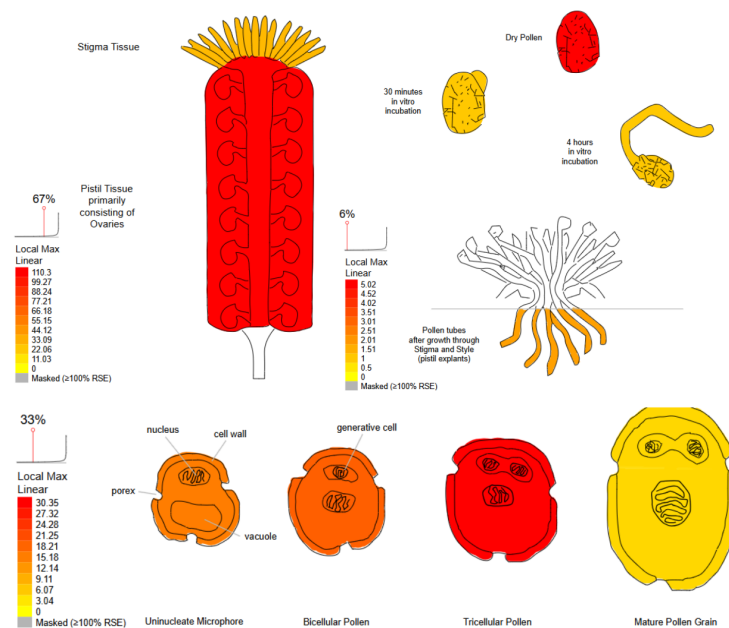


Fig. 12. Predicted expression pattern of *AtYAB3* in the female reproductive tissues, pollen development and pollen grain during *in vitro* incubation, germination and PT growing, obtained from the Arabidopsis eFP browser database.

3.3. Obtention of the *pAtERF114::GUS/YFP*, *pAtYAB3::GUS*, *p35S::AtERF114* and *p35S::AtYAB3* by Gateway Cloning

3.3.1. Obtention of the promoter and coding sequences of *AtERF114* and *AtYAB3*

The promoter and coding sequences of *AtERF114* and *AtYAB3* were amplified by PCR using genomic DNA (gDNA) extracted from Arabidopsis leaf tissue as a template. The resulting fragments were separated by agarose gel electrophoresis to confirm the presence of bands of the expected sizes (*AtERF114* - 1164 bp; *AtYAB3* - 2344 bp; *pAtERF114* - 1622 bp; *pAtYAB3* - 2870 bp) (Fig. 13). Bands corresponding to the target fragments were excised from the gel and purified for subsequent cloning procedures.

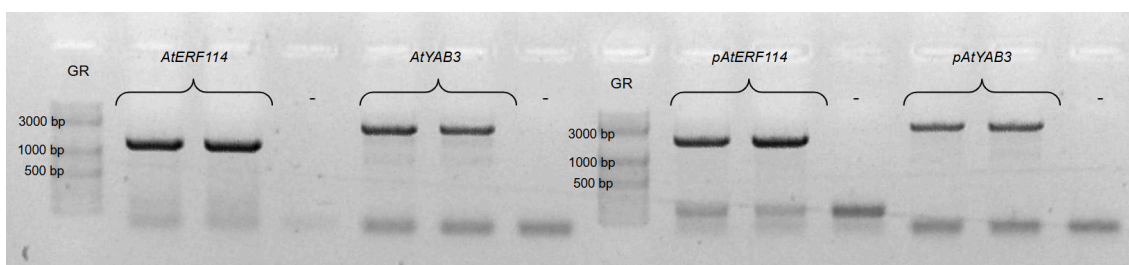


Fig. 13. Electrophoretic separation of the PCR products corresponding to the coding sequences of *AtERF114*, *AtYAB3*, and promoter sequences of *pAtERF114* and *pAtYAB3*. (-): negative control; GR: Gene Ruler (molecular size marker – 1Kb Plus DNA Ladder, ThermoScientific).

3.3.2. Obtention of the *pDONR207::AtERF114*, *pDONR207::AtYAB3*, *pDONR207::pAtERF114* e *pDONR207::pAtYAB3* constructs

The promoter and coding sequences of *AtERF114* and *AtYAB3* were inserted into the entry vector *pDONR207* (Supplementary material Figure 32) using the BP reaction of the Gateway cloning system. The products of this reaction were used to transform *E. coli* via electroporation. Positive colonies were identified by colony PCR using the same primer pairs used for the initial amplifications of these sequences, resulting in four positive colonies for each construct (Fig. 14). Two positive colonies per construct were cultured in liquid LB medium, and plasmid DNA was extracted by miniprep. These constructs were then subjected to restriction analysis to verify the correct orientation of the inserts (Fig. 15), which confirmed the expected band patterns for all tested constructs. Finally, the constructs were sequenced and aligned with the reference sequences to confirm the absence of mutations within the regions of interest (Supplementary material Figure 42-45). Although sequencing revealed a mutation in the coding sequences of *AtERF114* and *AtYAB3*, these were confirmed to be silent mutations that did not alter the encoded amino acid sequences. Therefore, these constructs were used in the subsequent experiments.

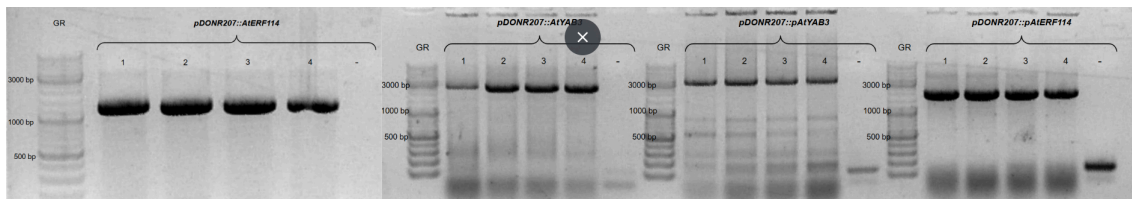


Fig. 14. Electrophoretic separation of the colony PCR results for *pDONR207::AtERF114*, *pDONR207::AtYAB3*, *pDONR207::pAtYAB3* and *pDONR207::pAtERF114*. 1-4: tested colonies for each construct; (-): negative control; GR: Gene Ruler (molecular size marker – 1Kb Plus DNA Ladder, ThermoScientific).

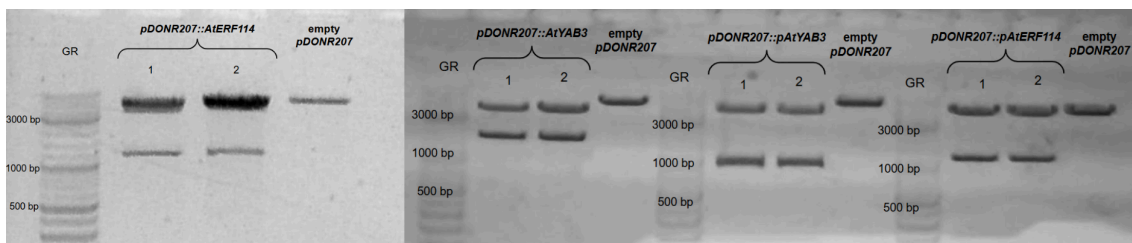


Fig. 15. Electrophoretic separation of the restriction analysis. The restriction enzymes used for *pDONR207::AtERF114* were EcoRV + KpnI (3417 and 1059 bp); for *pDONR207::AtYAB3* was EcoRV (4067 and 1589 bp); for *pDONR207::pAtYAB3* was EcoRV (5046 and 1136 bp); and for *pDONR207::pAtERF114* was EcoRI + SacI (4025 and 909 bp). For all the restriction analysis, an empty *pDONR207* plasmid was cut with the same enzymes as a negative control. 1-2: tested constructs; GR: Gene Ruler (molecular size marker – 1Kb Plus DNA Ladder, ThermoScientific).

3.3.3. Obtention of the *pB2GW7::AtERF114*, *pB2GW7::AtYAB3*, *pBGWFS7::pAtERF114*, *pBGWFS7::pAtYAB3* e *pMT589::pAtERF114* constructs

Using the LR reaction of the Gateway cloning system, the fragments of interest previously cloned into the entry vector *pDONR207* were transferred into the desired expression vectors. The coding sequences of the target genes were inserted into vector *pB2GW7* (Supplementary material Figure 33), which contains the constitutive promoter *p35S*, resulting in the *pB2GW7::AtERF114* and *pB2GW7::AtYAB3* constructs. The promoter regions of these genes were inserted into the vectors *pBGWFS7* and *pMT589* (Supplementary material Figure 34 and 35), which contain the coding sequences for *GUS* and *YFP*, respectively, in order to generate the *pBGWFS7::pAtERF114*, *pBGWFS7::pAtYAB3* and *pMT589::pAtERF114* reporter lines.

The products from the LR reactions were used to transform *E. coli* by electroporation. Positive colonies were identified by colony PCR using the same primer pairs used for

the initial amplification of the sequences (Fig. 16). Four positive colonies were obtained for *pB2GW7::AtERF114*, *pBGWFS7::pAtERF114* e *pB2GW7::AtYAB3*; one positive colony for *pMT589::pAtERF114*; and three positive colonies for *pBGWFS7::pAtYAB3*. Two positive colonies per construct were cultured in liquid LB medium, and plasmid DNA was extracted by miniprep. These constructs were then subjected to restriction analysis to verify the correct orientation of the inserts (Fig. 17), which confirmed the expected band patterns for all tested constructs. Finally, the constructs were sequenced and aligned with the reference sequences to confirm the absence of mutations within the regions of interest (Supplementary material Figure 46-50).

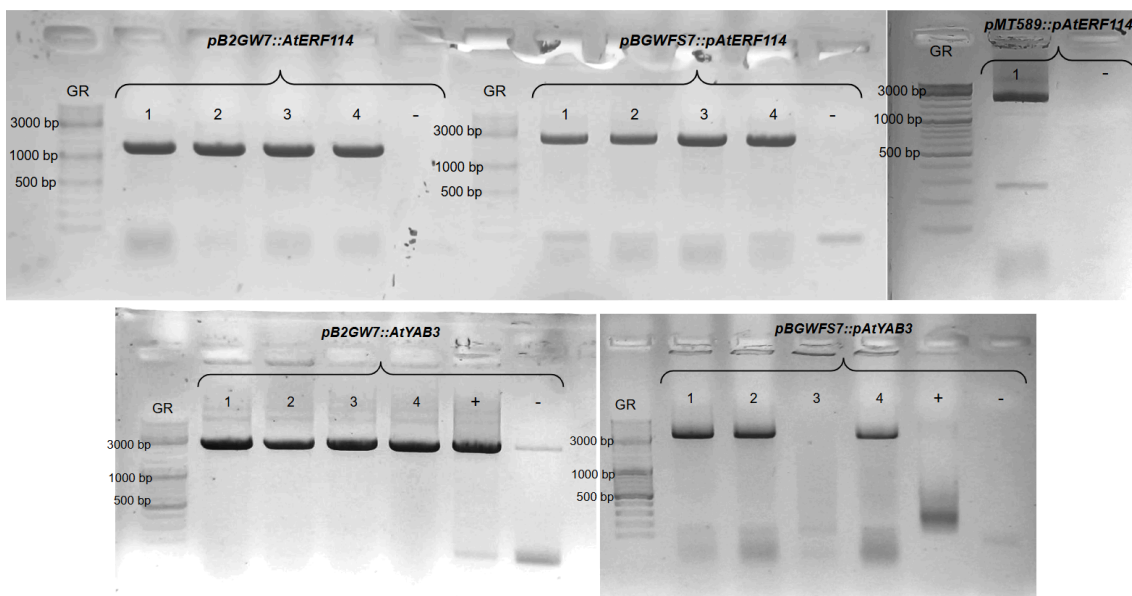


Fig. 16. Electrophoretic separation of the colony PCR results for *pB2GW7::AtERF114*, *pBGWFS7::pAtERF114*, *pMT589::pAtERF114*, *pB2GW7::AtYAB3* and *pBGWFS7::pAtYAB3*. 1-4: tested colonies for each construct; (+): positive control; (-): negative control; GR: Gene Ruler (molecular size marker – 1Kb Plus DNA Ladder, ThermoScientific).

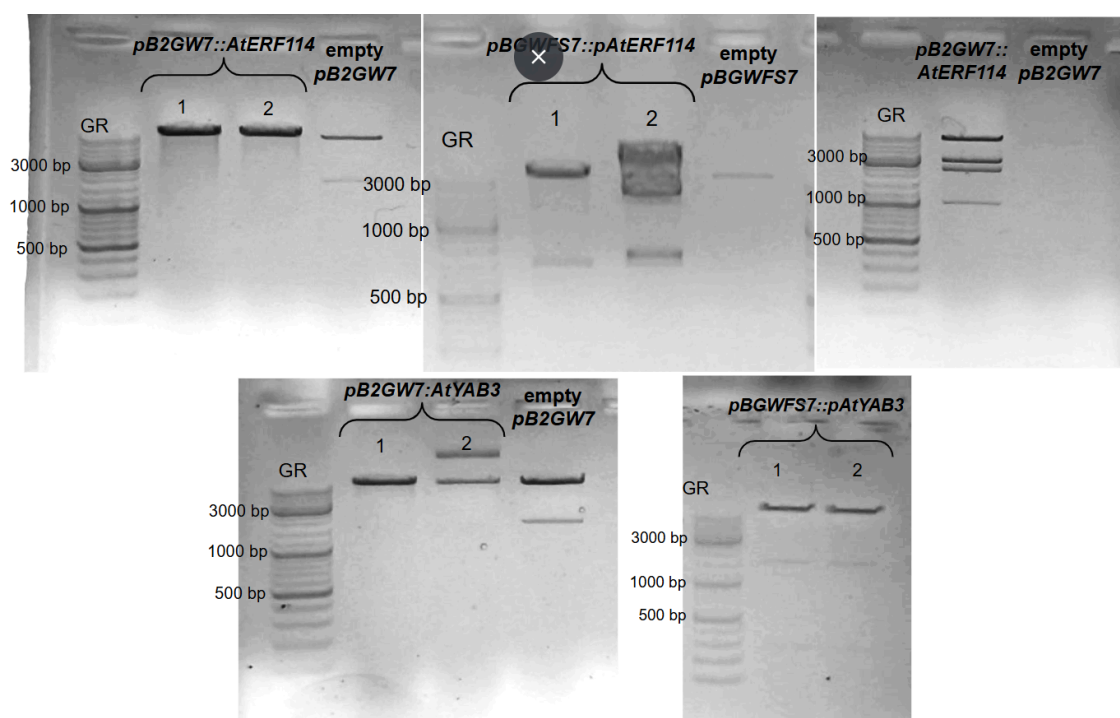


Fig. 17. Electrophoretic separation of the restriction analysis. The restriction enzymes used for *pB2GW7::AtERF114* were *Apal* + *KpnI* (9901 and 432 bp); for *pBGWFS7::pAtERF114* were *EcoRI* + *XbaI* (10942 and 1418 bp); for *pMT589::pAtERF114* were *EcoRI* + *EcoRV* (6759, 2894, 2094 and 897 bp); for *pB2GW7::AtYAB3* were *Apal* + *EcoRI* (11513 bp); and for *pBGWFS7::pAtYAB3* were *EcoRI* + *XbaI* (12068, 1331 and 309 bp). For all the restriction analysis, the respective empty plasmid was cut with the same enzymes as a negative control. 1-2: tested constructs; GR: Gene Ruler (molecular size marker – 1Kb Plus DNA Ladder, ThermoScientific).

3.3.4. Transformation of *A. tumefaciens*

Finally, the obtained constructs were used to transform *A. tumefaciens* via electroporation. The resulting colonies were screened by colony PCR to confirm the presence of the constructs (Fig. 18). Three positive colonies for *pB2GW7::AtERF114* and four positive colonies for *pB2GW7::AtYAB3* were obtained. The recombinant *A. tumefaciens* strains were subsequently used to transform *A. thaliana* plants by floral dip.

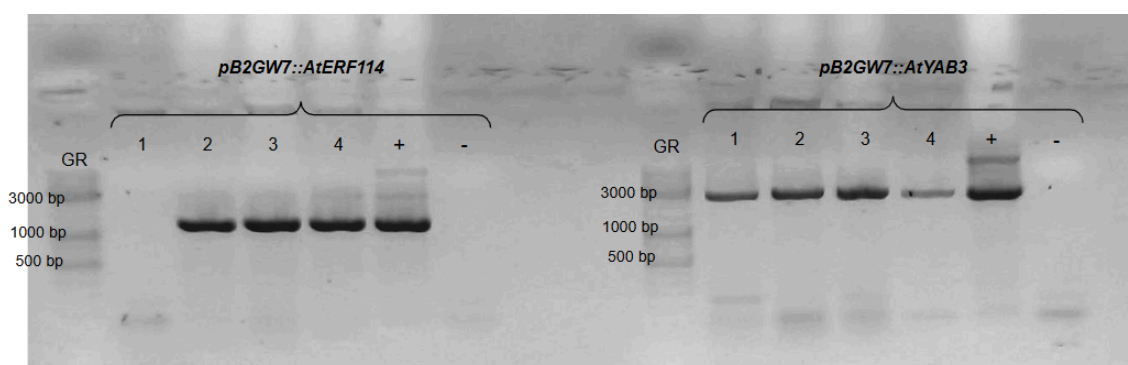


Fig. 18. Electrophoretic separation of the colony PCR results for *pB2GW7::AtERF114* and *pB2GW7::AtYAB3*. 1-4: tested colonies for each construct; (+): positive control; (-): negative control; GR: Gene Ruler (molecular size marker – 1Kb Plus DNA Ladder, ThermoScientific).

3.4. Obtention of the *p35S::OsERF98* and *p35S::OsBIP2* constructs by TEDA cloning

3.4.1. Obtention of the coding sequences of *OsERF98* e *OsBIP2*

The coding sequences of *OsERF98* and *OsBIP2* were amplified by PCR using gDNA extracted from *Oryza sativa* leaf tissue. The resulting fragments were separated by agarose gel electrophoresis to verify the band's size (*OsERF98* - 939 bp; *OsBIP2* - 2561 bp) (Fig. 19). Bands corresponding to the target fragments were excised from the gel and purified for subsequent cloning procedures.

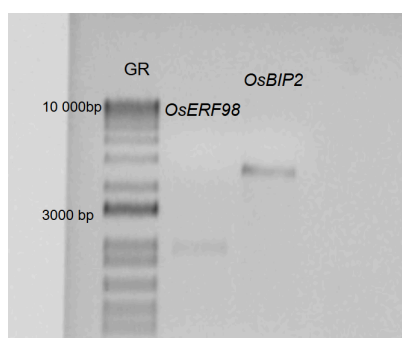


Fig. 19. Electrophoretic separation of the PCR products corresponding to the coding sequences of *OsERF98* and *OsBIP2*. GR: Gene Ruler (molecular size marker - GeneRuler 1kb DNA Ladder (Thermo fisher™)).

3.4.2. Linearization of the *pBY02* vector

Prior to the TEDA cloning reaction, the *pBY02* vector was linearized using the restriction enzymes *Sall* (New England Biolabs™) and *Acc65I* (Thermo Fisher™) to generate 3'-overhanging cohesive ends. Linearization was confirmed by agarose gel electrophoresis (Fig. 20). As the digested vector adopts a linear conformation, it migrates more slowly through the gel compared to the undigested supercoiled plasmid, which exhibits faster migration due to its compact structure.

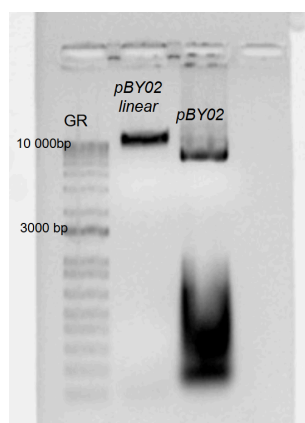


Fig. 20. Electrophoretic separation of the linearized and non-linearized *pBY02* vector. GR: Gene Ruler (molecular size marker - GeneRuler 1kb DNA Ladder (Thermo fisher™)).

3.4.3. Obtention of the *pBY02::OsERF98* e *pBY02::BIP2* constructs

The coding sequences of *OsERF98* and *OsBIP2* were inserted into the previously linearized vector *pBY02* (Supplementary material Figure 36). The resulting constructs were used to transform *E. coli*. Positive colonies were identified by colony PCR using the same primer pairs used for the initial amplification of these sequences (Fig. 21), resulting in four positive colonies for *pBY02::OsERF98* and two for *pBY02::OsBIP2*. Two positive colonies per construct were cultured in liquid LB medium, and plasmid DNA was extracted by miniprep. These constructs were then subjected to restriction analysis to verify the correct orientation of the inserts (Fig. 22), which confirmed the expected band patterns for all tested constructs. Finally, the constructs were

sequenced and aligned with the reference sequences to confirm the absence of mutations within the regions of interest (Supplementary material Figure 51 and 52).

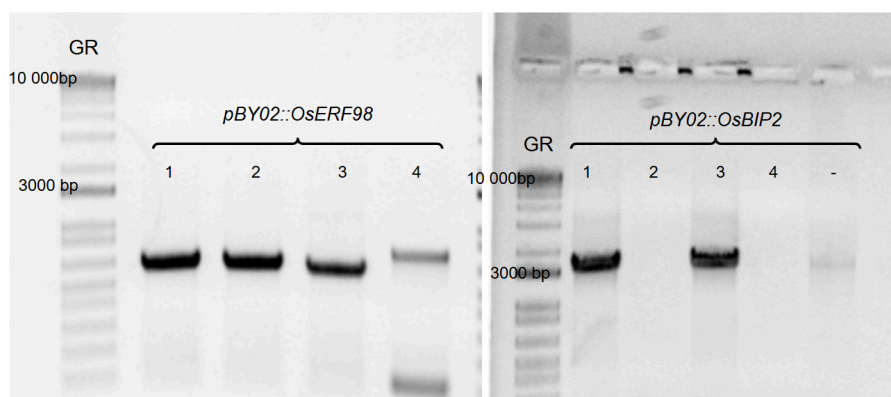


Fig. 21. Electrophoretic separation of the colony PCR results for *pBY02::OsERF98* and *pBY02::OsBIP2*. 1-4: tested colonies for each construct; (-): negative control; GR: Gene Ruler (molecular size marker - GeneRuler 1kb DNA Ladder (Thermo fisher™)).

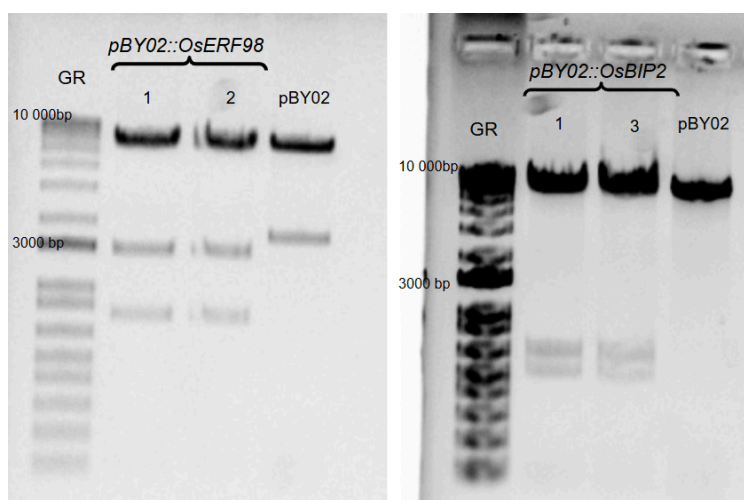


Fig. 22. Electrophoretic separation of the restriction analysis. The restriction enzyme used for *pBY02::OsERF98* was AatII (9778, 2286 and 1164 bp); for *pBY02::OsBIP2* was SacI (12785, 1081, 852 and 132 bp). For all the restriction analysis, an empty *pBY02* plasmid was cut with the same enzymes as a negative control. 1-2: tested constructs; GR: Gene Ruler (molecular size marker - GeneRuler 1kb DNA Ladder (Thermo fisher™)).

3.5. Obtention of the Prime Editing construct for introduction of an heat-activated switch in the *OsYABBY7* gene promoter

3.5.1. *OsYABBY7* promoter analysis

To determine the most suitable site for the insertion of the bio-switch within the promoter of *OsYABBY7*, a sequence analysis was performed to avoid regions containing active cis-regulatory elements (Supplementary material Figure 41). Based on this analysis, a region devoid of active regulatory elements was selected and used as input into the PlantPegDesigner tool (<http://www.plantgenomeediting.net/>) to identify the most appropriate spacer (Fig. 23). The linker sequence was designed using the pegLIT software (<https://peglit.liugroup.us>).

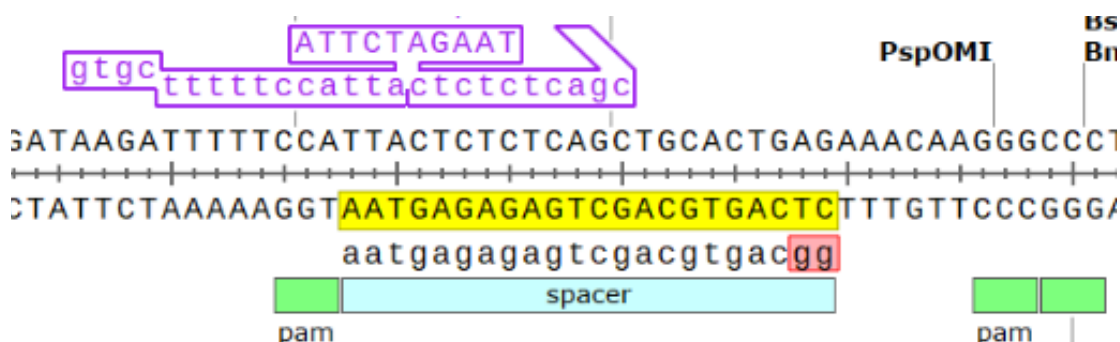


Fig. 23. Schematic representation of the promoter region of *OsYABBY7* targeted for the introduction of the bio-switch ATTCTAGAAT, indicating the binding sites of the spacer (in blue) and the extension (in purple). The PAM sites are marked in green.

3.5.2. Obtention of the PE construct

To obtain the spacer and extension sequences for insertion into the *pAG731* plasmid, two pairs of complementary primers were designed for each sequence, with modified ends to generate overhangs compatible with those of the plasmid when digested with the restriction enzyme *BsaI*-HF. The primers were annealed by incubation to form double-stranded DNA fragments. These sequences were then inserted into the plasmid via GoldenGate assembly. The resulting reaction products were used to transform *E. coli* by heat shock. Positive colonies were identified by the absence of fluorescence and confirmed by colony PCR (Fig. 24) using the primer pair 33 (spacer forward

primer) and 50 (Supplementary material Table 23). Three positive colonies were obtained, which were cultured in liquid LB medium. The plasmids were extracted by miniprep and subjected to restriction digestion to verify the presence of the inserts (Fig. 25). Since the vector *pAG731* suffered some modifications in the lab, the sequence map was outdated and the resulting bands did not correspond to the expected ones, so the presence of the inserts was made only by comparison with the empty vector. Colonies 1 and 2 exhibited banding patterns distinct from that of the empty vector, indicating the presence of the inserts. Finally, the constructs were sequenced and aligned with the expected sequences to confirm the absence of mutations in the regions of interest (Supplementary material Figure 53).

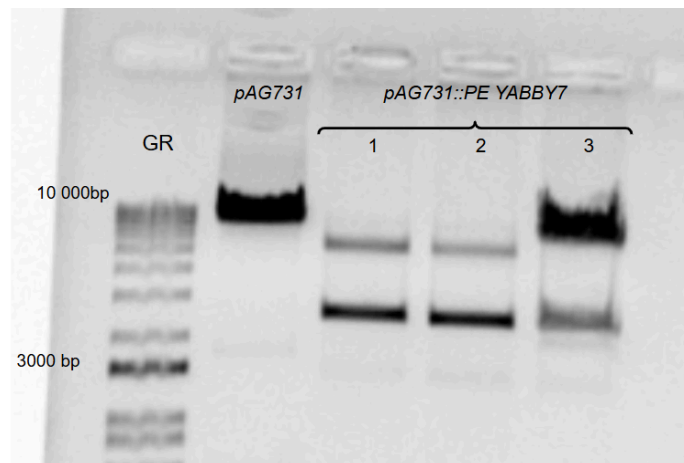


Fig. 24. Electrophoretic separation of the restriction analysis. The restriction enzyme used for *pAG731::PE YABBY7* was PflMI. For all the restriction analysis, an empty *pAG731* plasmid was cut with the same enzymes as a negative control. 1-3: tested constructs; GR: Gene Ruler (molecular size marker - GeneRuler 1kb DNA Ladder (Thermo fisher™)).

3.6. Expression pattern of *AtYAB3*

Plants obtained from *A. thaliana* transformation with the *pAtYAB3::GUS* construct were analyzed to determine the expression pattern of the corresponding protein in reproductive tissues. Both BASTA selection and genotyping of the transformed plants were performed by another member of the laboratory during my stay in the USA.

The YAB3 promoter guided GUS expression in the anthers and in the stigmatic cells of the pistil. No GUS activity was detected in the transmitting tissue or in the ovules (Fig. 25).

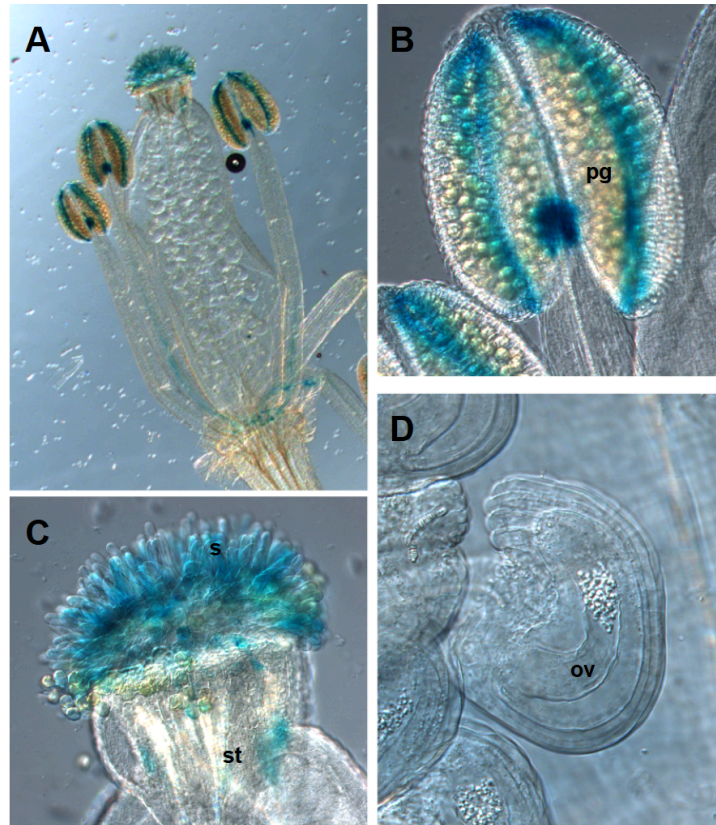


Fig. 25. Histochemical analysis of GUS activity in pistils and anthers of *A. thaliana* transformed with the *pAtYAB3::GUS* construct. **A-D** - GUS activity driven by the *YAB3* promoter in reproductive tissues of Arabidopsis: **A** - whole flower, **B** - anther, **C** - stigma. **D** - Absence of GUS activity in ovules. (pg) - pollen grain; (s) - stigma; (st) - style; (ov) - ovule.

3.7. Phenotypic analysis of the *yab3* knock-down mutant

3.7.1. Confirmation of the T-DNA insertion

In order to select the homozygous (HM) plants for the T-DNA insertion from the *yab3* (SALK_086776) line, several genotyping PCRs were performed. Three primers were used. The primers LP (1803) and RP (1804) (Supplementary material Table 23) amplify the WT band (1101 bp) in the absence of the T-DNA insertion. If the insertion is

present, this WT band is not amplified. The BP primer (LBb3.1), which is specific to the T-DNA insertion, amplifies the mutant (mt) band in combination with primer 1804 (498-798 bp). Plants that showed only the mt band were considered HM for the insertion, while those with both WT and mt bands were considered heterozygous (HZ). Plants that displayed only the WT band were considered WT, indicating the absence of the insertion in both alleles. The primer pair 1803 + LBb3.1 was also tested to confirm the absence of a secondary T-DNA insertion within the gene. No amplification was detected with this pair.

Genotyping results were analysed by agarose gel electrophoresis (Fig. 26). Plants 1, 2, 3, 6, 8, 11, 12, 14, 15, 16 and 17 were identified as HM for the T-DNA insertion, plants 7 and 9 as HZ and plants 4 and 5 as WT.

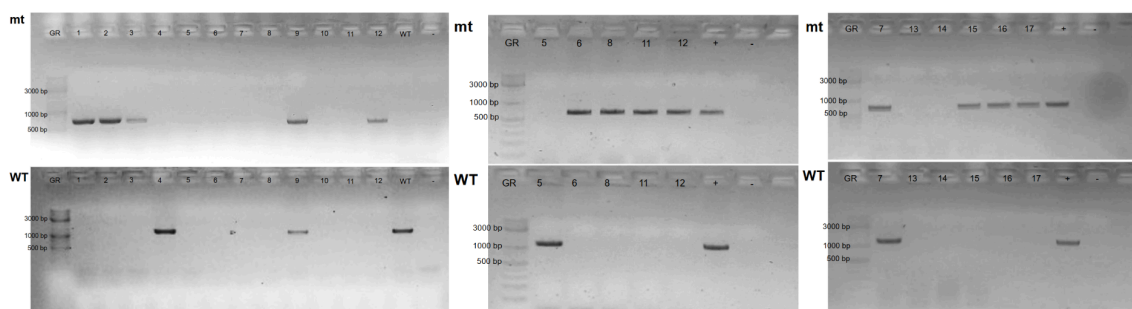


Fig. 26. Electrophoretic separation of the genotyping PCR results for *yab3* mutant plants. mt: mutant band, amplified using the primers 1803 + LBb3.1; WT: wild type band, amplified using the primers 1803 + 1804. 1-17: tested plants; (+): positive control; (-): negative control; GR: Gene Ruler (molecular size marker – 1Kb Plus DNA Ladder, ThermoScientific).

To determine the precise location of the T-DNA insertion in the *yab3* line, the mt band amplified using the primer pair 1804 + LBb3.1 was excised from the agarose gel, purified and sent for sequencing (Supplementary material Figure 54). This analysis confirmed that the T-DNA is inserted at the genomic site predicted by T-DNA express data (Fig. 27).

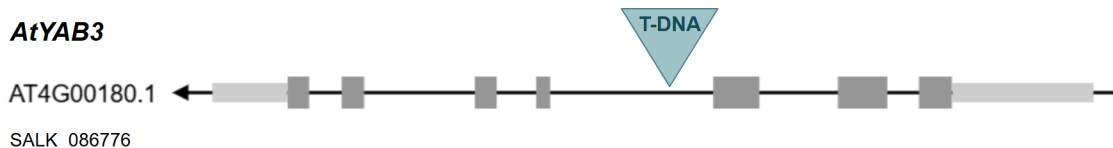


Fig. 27. T-DNA insertion site in the *yab3* line, indicated by a blue triangle. Rectangles represent exons, and lines represent the non-coding regions of the gene.

The subsequent phenotypic analysis were performed only in HM *yab3* plants.

3.7.2. Characterization of gene expression in *A. thaliana* mutant lines

After confirming the homozygous plants for this gene, RT-qPCR was performed to assess whether these lines are knock-out/down for *AtYAB3*, by comparing its expression under normal conditions between WT and mutant plants (Fig. 28). The primer pair used showed efficiency values within the 80-120% range, with correlation coefficients (R^2) above 0.990, ensuring the linearity of the calibration curve obtained from serial cDNA dilutions (Supplementary material Figure 40). Melt curve analysis revealed a single peak, confirming the absence of non-specific amplification (Supplementary material Figure 40 B).

A slight reduction in *AtYAB3* expression was observed in this line. However, the difference was not statistically significant. Nevertheless, the reproductive performance of this line was analyzed under both normal and HS conditions.

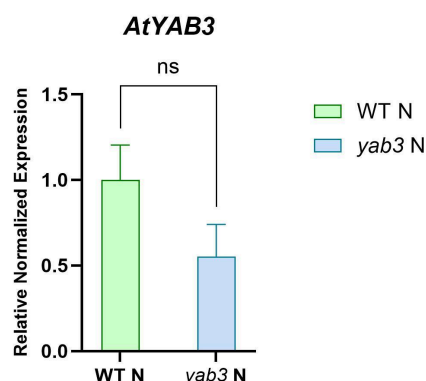


Fig. 28. Relative expression of *AtYAB3* in WT and *yab3* plants. A non-significant reduction in *yab3* expression was observed in this mutant line. Expression levels were normalized to the reference genes *AtACT8* and *AtRUB1*. Each bar represents the mean of three biological replicates. (ns) - non-significant difference according to Sidak test ($p \leq 0.05$).

3.7.3. Reproductive performance under control and HS conditions

3.7.3.1. Pollen tube growth analysis

Pollen tube growth and callose deposition along the pistil tissues were visualized using aniline blue staining, allowing detailed analysis of PT behavior and rates of ovule fertilization. Aniline blue staining was performed on self-pollinated pistils from both WT and *yab3* plants, to compare the percentage of ovules receiving PTs under control (N) and HS conditions. The number of ovules with and without receiving one PT was quantified and statistically compared using Tukey test to determine whether the *yab3* mutant line responds differently to HS conditions.

In WT plants, although the number of pollen grains on the stigma remained comparable between treatments (Fig. 29 A, D), PT growth under HS conditions was impaired. PT showed reduced ability to reach the ovules, resulting in a decrease in the percentage of fertilized ovules from 75.62% under control conditions to 60.08% under HS (Fig. 29 G). Moreover, HS led to an increased number of ovules displaying internal callose deposition (Fig. 29 F).

In *yab3* mutant plants, high numbers of pollen grains were also observed on the stigma. However, under normal conditions, these plants already exhibited a reduced percentage of fertilized ovules compared to WT (Fig. 30 A). Additionally, some ovules

in this mutant line showed callose deposition even under control conditions. Under HS, there was no apparent difference in pollen grain number deposited in the stigma (Fig. 30 D), but the percentage of fertilized ovules further decreased to 51.34% (Fig. 30 G), indicating a reduced capacity of PTs to reach the ovules in the HS conditions.

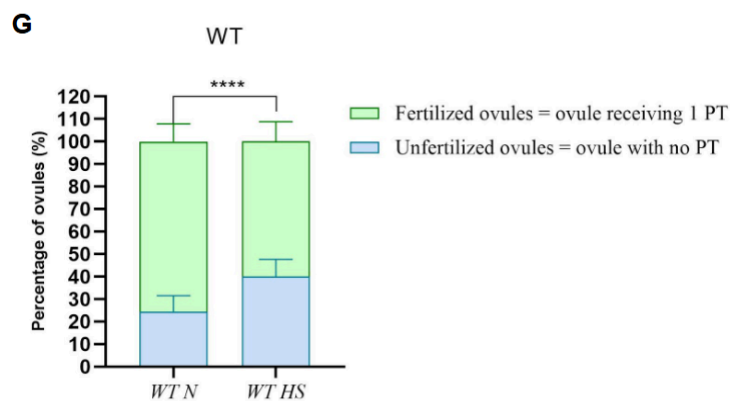
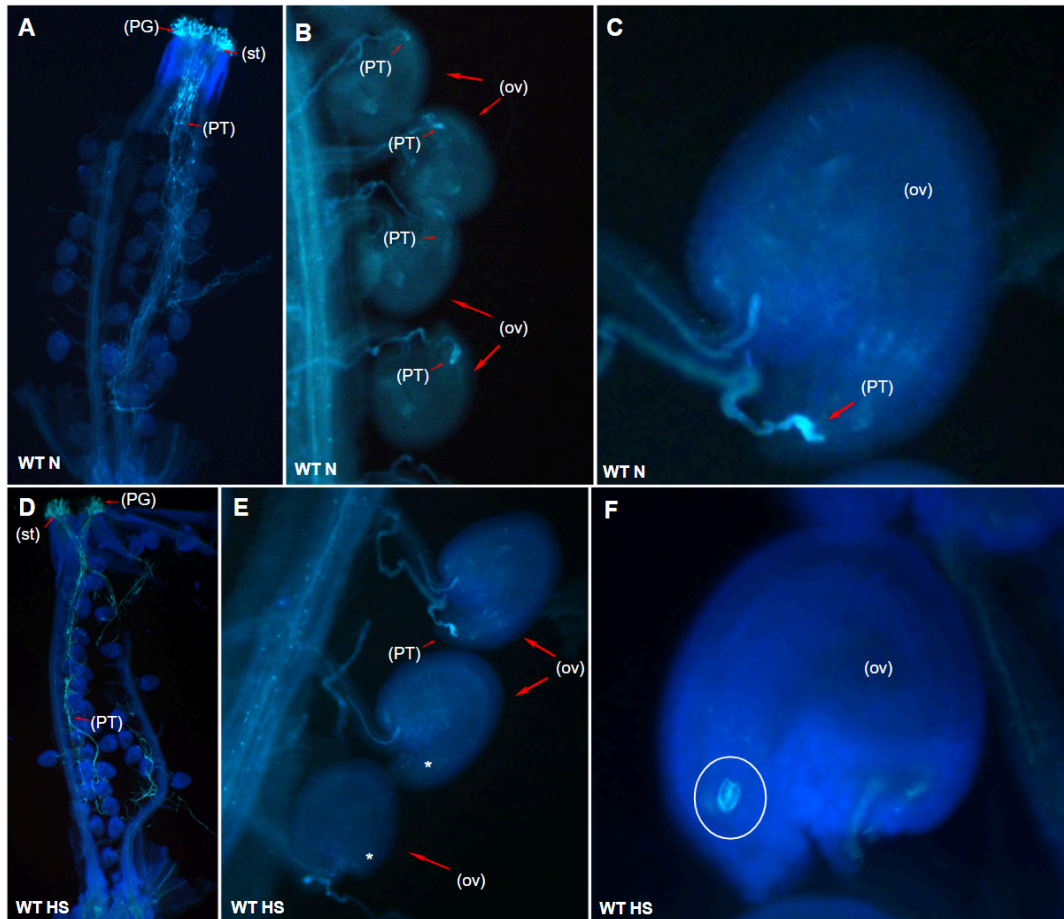


Fig. 29. Aniline blue staining of self-pollinated pistils from WT flowers under control (N) and HS conditions, and quantitative analysis of fertilized ovules (receiving a PT at the micropylar region) and unfertilized ovules (not receiving a PT) under both conditions. A, B, C - control conditions (N); D, E, F - HS conditions. A - Pollen grains deposited on the stigma surface and PT growth through the pistil tissues toward the ovules in control conditions; B - PTs reaching the micropylar region of ovules; C - Detail of an ovule receiving a PT at its micropylar region; D - Pollen grains on the stigma surface and PT growth through the pistil tissues toward the ovules in HS conditions; E - One ovule receiving a PT and two ovules without PT entering through the micropyle (indicated with asterisks); F - Detail of an ovule without PT entering through the micropyle, showing callose accumulation in this region (circled). (PG) - pollen grain; (st) - stigma; (PT) - pollen tube; (ov) - ovule. G - Percentage of fertilized and unfertilized ovules under control (n = 275) and HS (n = 261) conditions. Error bars represent mean $\pm$ standard deviation, **** above bars indicate significant differences according to Tukey test ($p < 0,0001$).

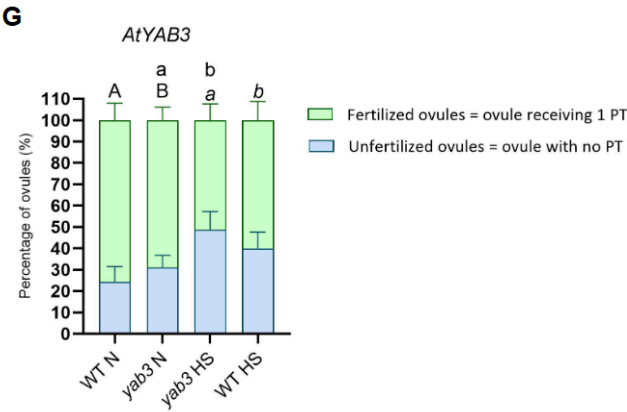
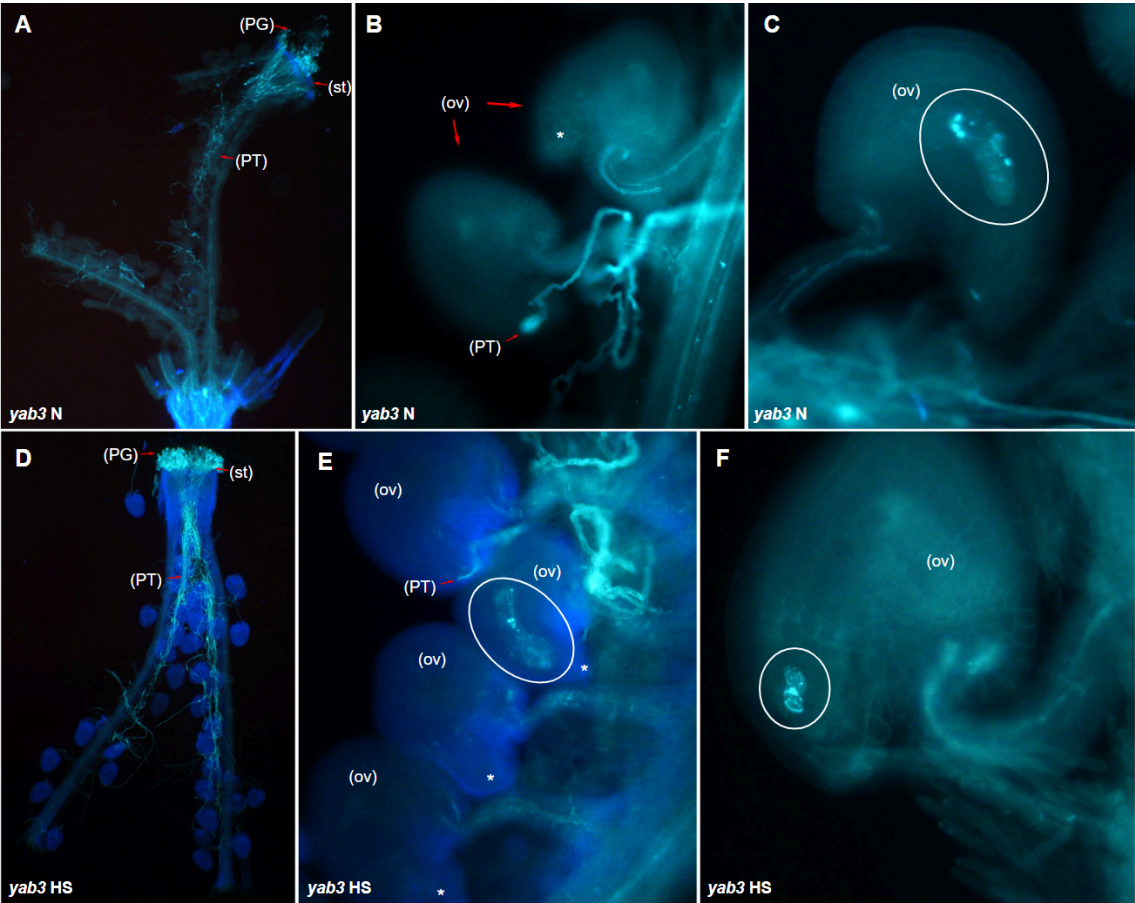


Fig. 30. Aniline blue staining of self-pollinated pistils from *yab3* flowers under control (N) and HS conditions, and quantitative analysis of fertilized ovules (receiving a PT at the micropylar region) and unfertilized ovules (not receiving a PT) under both conditions. A, B, C - control conditions (N); D, E, F - HS conditions. A - Pollen grains on the stigma surface and PT growth through the pistil tissues toward the ovules in control conditions B - PTs reaching the micropylar region of one ovule, and another ovule lacking PT at its entrance marked with an asterisk; C - Detail of an ovule without PT entering the micropyle, showing callose accumulation in the whole embryo sac region (circled); D - Pollen grains deposited on the stigma and PT growth toward the ovules in HS conditions; E - One ovule receiving a PT and three ovules without PT entry at the micropyle (marked with asterisks). One of the unfertilized ovules shows callose accumulation in the embryo sac region (circled). (PG) - pollen grain; (st) - stigma; (PT) - pollen tube; (ov) - ovule. G - Percentage of fertilized and unfertilized ovules in WT under control conditions (N) (n = 275); *yab3* under control conditions (N) (n = 258), *yab3* under HS conditions (n = 275) and WT under HS conditions (n = 261). Error bars denote mean $\pm$ standard deviation, the letters A and B above bars indicate significant difference between WT N and *yab3* N, the letters a and b above bars indicate significant difference between *yab3* N and *yab3* HS, and the letters *a* and *b* indicate significant difference between *yab3* HS and WT HS according to Tukey test ($p < 0,0001$).

3.7.3.2. Clearing of siliques

Siliques from WT and *yab3* mutant plants obtained from flowers under control (N) and HS conditions were cleared and observed under a magnifying glass (Fig. 31 A - D). Overall, siliques formed under HS conditions exhibited a higher frequency of seed abortion and greater variability in size. However, no apparent differences were observed between siliques from *yab3* and WT plants under either condition. As a result, no further seed set analysis was conducted.

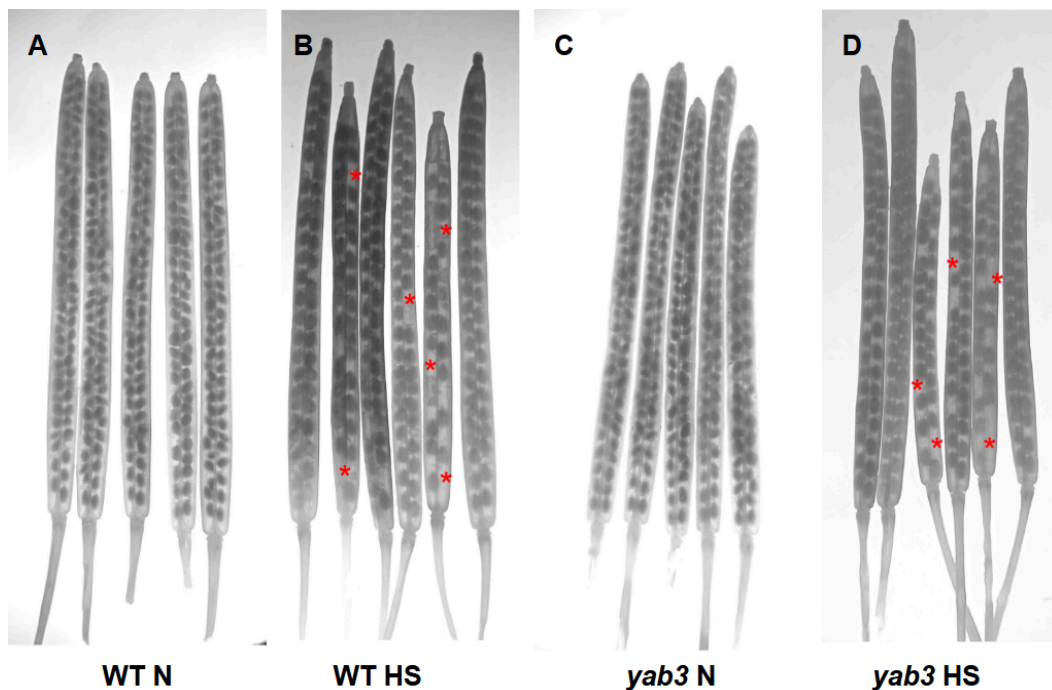


Fig. 31. Ethanol-fixed siliques observed under a magnifying glass. A - WT siliques under control (N) conditions. B - WT siliques developed from flowers subjected to HS. C - *yab3* siliques under control (N) conditions. D - *yab3* siliques developed from flowers subjected to HS. The red asterisks indicate empty spaces, indicating seed formation failure inside these siliques.

4. Discussion

The emergence of agriculture marked a fundamental shift in human society, enabling sedentary lifestyles by allowing communities to produce their own food. Plants are essential in this context, as they serve as sources of food, raw materials, pharmaceuticals and much more (Dubey and Prakash, 2021). However, climate change and the rapid increase in global average temperatures are already exerting significant impacts on various crops, leading to reduced productivity (Tu *et al.*, 2022). Therefore, understanding the effects of this abiotic stress on plants, particularly during the reproductive phase, is becoming increasingly important, as fruits and seeds are of critical agricultural and economic value. With the advancement of science and the emergence of gene editing techniques, it is now possible to introduce new genes or modify gene expression levels with precision and efficiency, offering promising

strategies to enhance plant resilience to HS (Khalil, 2020). Nonetheless, a prerequisite for these interventions is a comprehensive understanding of the existing tolerance mechanisms, and the identification of genes associated with beneficial traits that enable plant growth under increasingly adverse environmental conditions.

4.1. Gene expression profile for the candidate genes in *O. sativa*

For this study, the genes *OsERF98*, *OsBIP2* and *OsYABBY7* were selected from a list of differentially expressed genes under HS conditions in the reproductive phase of the heat-tolerant rice variety N22. According to this list, obtained from a previously conducted RNA-seq analysis (González-Schain *et al.*, 2016), these genes are highly upregulated in response to HS in this variety. These genes were subjected to a literature review in order to determine if their expression is specific to the reproductive tissues, and whether there is existing literature associating them with stress-related mechanisms. We found out that *OsERF98* is predominantly expressed in seeds and anthers. Although it has not been studied in relation to HS, it belongs to the same family of *OsERF115*, which plays a role in HS and drought tolerance. *OsBIP2* is expressed in both male and female reproductive tissues and has already been associated with thermotolerance of the PT. *OsYABBY7* is mainly expressed in female reproductive tissues and, although it has not been studied in rice, it has orthologs in soybean and cotton that have been implicated in responses to drought and salinity stress. Based on these observations, we hypothesized that they may be involved in the plant's response mechanism to HS.

We started by performing a RT-qPCR to confirm the overexpression of these genes in N22. However, the design and optimization of primers for this technique can be time-consuming, due to several factors that influence primer quality, such as the size of the amplified sequence, the formation of secondary structures, GC content, and cDNA concentration, among others. As a result, it was not possible to obtain primer pairs for *OsERF98* and *OsBIP2* that met the desired quality parameters within the time frame of this study, and optimization is still ongoing. Consequently, gene expression analysis could only be performed for *OsYABBY7*. Nevertheless, due to the limited time available for construct generation for rice, the constructs for these genes (*OsERF98* and *OsBIP2*) were prepared even in the absence of RT-qPCR validation.

For *OsYABBY7*, its overexpression under HS conditions was confirmed, consistent with the RNA-seq data. The expression pattern of this gene was also analyzed in a heat-sensitive rice varieties - Nipponbare (Nipp) - and a cold tolerant variety - Kitaake (Kit). It was initially expected that gene expression in the sensitive varieties would contrast more with that observed in the tolerant N22. However, it was found that even in the sensitive varieties there is a significant increase in *OsYABBY7* expression under HS conditions. Nevertheless, the magnitude of induction in N22 remains markedly higher than in the other tested varieties. This suggests that *OsYABBY7* may still play a role in the heat-tolerance mechanism, as the highest expression increase is observed in the most tolerant variety. Therefore, *OsYABBY7* appears to be a promising candidate for phenotypic analysis under HS, in order to elucidate its potential role in HS response regulation.

Although the results obtained for *OsYABBY7* were promising, it remains necessary to repeat the HS assay to verify the consistency of these results. Additionally, it would be important to complete the primer optimization for the remaining genes and assess their expression patterns in the three rice varieties under study.

4.2. Gene expression profile for the candidate genes in *A. thaliana*

Since the study of candidate genes in rice is highly time consuming for obtaining results within the project timeline, Arabidopsis was used as proof of concept to study the rice orthologues' phenotype. This model plant has proven to be a valuable tool for studying the most promising genes, as the results can often be effectively translated to other crops, including rice.

The gene expression profiles of *AtERF114* and *AtYAB3*, orthologues of *OsERF98* and *OsYABBY7*, respectively, were analysed to confirm whether these genes exhibit expression patterns similar to those observed in *O. sativa*, and consequently, to assess whether they may perform analogous functions in both species.

The results obtained for *AtYAB3* showed that, similar to rice, its expression increases under HS conditions. However, unlike rice, this increase was not statistically significant. This reduced expression response may be related to the Arabidopsis variety used (Columbia-0 ecotype, Col-0), and maybe other Arabidopsis exotypes exhibit a stronger induction of these genes' expression. Previous studies have demonstrated that this

ecotype displays a lower number of differentially regulated transcripts under HS (Barah *et al.*, 2013). Therefore, the nonsignificant results observed for *AtYAB3* may not indicate a lack of involvement of this gene in the tolerance mechanism, but rather reflect the higher sensitivity of Col-0 to HS. It would be worthwhile to repeat this study using multiple *Arabidopsis* ecotypes with varying levels of HS tolerance to clarify whether the less consistent results compared to those seen in rice are due to the ecotype used or may indicate a functional difference of the gene.

For *AtERF114*, it was observed that its expression significantly decreases under HS, with almost no expression detected in these conditions. This result contrasts with the RNA-seq data for *OsERF98*, whose expression increases under elevated temperatures. Such a marked difference in expression profiles suggests a divergent HS response between the orthologues, indicating that the function of *AtERF114* may differ from that of its orthologue in rice.

4.3. Gene expression characterization in *Arabidopsis* mutant lines

The study of mutant lines provides valuable insights into the function of target genes. By comparing the phenotypes of mutant plants for a given gene with those of WT plants, it is possible to identify differences that serve as clues to infer the gene's role. To investigate the function of *AtYAB3*, a T-DNA insertion line with the insertion located in an intron of this gene was analyzed. Additional T-DNA insertion lines were ordered - one for the same gene with the insertion in the promoter region, and another for *AtERF114*. However, the seeds from these lines failed to germinate despite multiple attempts both in soil and *in vitro*. Due to the limited timeframe available for this project, it was not possible to reorder seeds in time, and therefore, only one of the three intended mutant lines could be analyzed.

After confirming the HM plants for the T-DNA insertion in *AtYAB3* and verifying the insertion site, a RT-qPCR was performed. The aim was to determine whether the insertion affected transcript levels of the target gene, either by reducing its expression or potentially silencing it. The results showed that, although there was a slight decrease in *AtYAB3* expression, the difference was not statistically significant. However, due to unavailability of additional lines for this gene, and considering that a modest reduction in expression was still observed, phenotypic analysis were carried out for this line to

assess whether the expression change had any impact on HS tolerance at the reproductive level.

4.4. Expression pattern of *AtYAB3*

Before initiating phenotypic studies on the *yab3* mutant line, first the expression pattern of *AtYAB3* in reproductive tissues was examined, to identify the specific sites where it is expressed and thereby guide the focus of our investigation for these tissues. For this purpose, the *pYAB3::GUS* reporter line was produced and used. This line contains the β -glucuronidase (GUS) coding sequence fused to the promoter of the *AtYAB3* gene, so GUS activity is detected in the same tissues and conditions where *AtYAB3* is normally expressed.

Using this approach, it was observed that in open *Arabidopsis* flowers, this gene is normally expressed in the anthers tissues (but not in the pollen grains) and in the stigmatic cells of the pistil. No GUS activity was detected in the transmitting tissue or the ovules. These results contrast with the data available in the *Arabidopsis* eFP Browser database, which indicates that *AtYAB3* is expressed throughout the pistil, with lower expression in the stigma compared to other pistil tissues. However, since the GUS expression patterns was consistent across multiple plants analyzed, it is possible that the database data were obtained from flowers at a different developmental stage. It is also possible that the promoter region used in this construct was not the ideal. Maybe it was necessary to use a bigger region, or include other regions that may have regulatory regions, like the introns.

When pollen reaches the stigma surface, the stigma provides it with water, nutrients and other small molecules to facilitate its hydration and germination. Thus, the stigma plays a critical role in the reproductive process. HS reduces pollen adhesion to the stigma, leading to a lower number of pollen grains that successfully germinate. Consequently, the number of PTs growing through the pistil is reduced, ultimately decreasing the number of fertilized ovules (Snider *et al.*, 2011; Peraudeau *et al.*, 2015). The high expression of *AtYAB3* in stigmatic cells may indicate that this gene is involved in pollen-pistil interactions at this level. If this is the case, then its overexpression under HS conditions could potentially serve as a compensatory mechanism to counteract the

reduced pollen adhesion under this condition. To investigate this hypothesis, we proceeded with phenotypic analysis of the *yab3* mutant line, focusing on reproduction-related traits.

4.5. The impact of HS in *yab3* mutant plants

The aniline blue staining technique is one of the most widely used methods for phenotypic characterization of the plant reproductive process, as it enables the visualization of multiple stages of fertilization. This includes pollen adhesion to the stigma since the dye stains callose present in pollen grains, the growth of PTs through pistil tissues including the formation of callose plugs during their elongation, and the entry of the PT into the ovule, which allows the identification of successful fertilization or defects such as the occurrence of polytubey. Additionally, this technique can also be used to assess ovule viability, as one of the indicators of a non-viable ovule is the accumulation of callose within it. Therefore, this method was employed to evaluate the reproductive process in both WT and *yab3* plants under normal and HS conditions.

In WT plants, exposure to HS during anthesis resulted in a reduced number of fertilized ovules and an increased number of ovules with callose accumulation, indicating loss of viability. The number of pollen grains adhered to the stigma did not appear to be affected. However, PTs were shorter and fewer in number, failing to reach the lower regions of the pistil. Since PTs require approximately 8 to 10 hours to reach the embryo sac (Kandasamy *et al.*, 1994), a recovery period of 24 hours was allowed before flower collection. Therefore, this difference cannot be attributed to the timing of sample collection, as sufficient time for PT growth was provided. These observations confirm that PT growth is significantly slower under HS conditions.

In *yab3* plants, the number of fertilized ovules was lower than in WT under control conditions, and decreased even further under HS, which is consistent with the hypothesis that this gene contributes to increased HS tolerance. However, a substantial number of ovules were still successfully fertilized. Although expression analysis showed that this gene is strongly expressed in the stigma, no noticeable differences were observed in the number of pollen grains adhering to this region, suggesting that pollen adhesion was not impaired. Similarly, the number of PT growing along the pistil tissues, did not differ significantly from that observed in WT.

Since unfertilized ovules were observed in both WT and *yab3* plants under both conditions, a preliminary analysis was conducted to investigate potential failures in seed formation within siliques. Siliques from each experimental condition were collected and cleared for microscopic observation. This technique renders silique tissues transparent, allowing the visualization of seed within the structure and the detection of empty spaces indicative of seed formation failure. Some seed development failures were observed in both plant lines when exposed to HS during anthesis, demonstrating the reproductive impact of HS. However, unlike what was observed in aniline blue staining, no significant differences were detected between *yab3* and WT plants. This suggests that although *yab3* plants exhibit a greater delay in PT growth, leading to a higher number of unfertilized ovules 24 hours after anthesis, the tubes may continue to grow and eventually reach the embryo sac, allowing double fertilization and seed formation, ultimately not compromising productivity.

Although the phenotypic differences observed were minor, this is likely due to the relatively modest reduction in *AtYAB3* expression in the mutant line used, meaning that the protein is still produced in these tissues and can therefore continue to perform its function. It is possible that a knockout line for *AtYAB3* would exhibit more pronounced defects, including a lower number of fertilized ovules and seeds formed, and potentially impairments in pollen reception. As such, the current results are not sufficient to draw definitive conclusions regarding the role of this gene in reproductive tissue HS tolerance or its apparent function. Future studies should focus on other T-DNA insertion lines with a more substantial reduction in *AtYAB3* expression, as well as the *AtYAB3* overexpression line generated in this work.

5. Conclusion and future perspectives

Although the phenotypic analysis of the mutant *yab3* line was not as conclusive as initially expected, it demonstrated greater sensitivity to HS compared to the WT variety, suggesting that it remains a promising candidate gene for the process under investigation. Throughout the course of this study, several constructs were also obtained, which, upon analysis, are expected to complement the current findings and potentially provide further insight into the roles of the target genes in the HS tolerance mechanism.

In addition, the results once again highlighted the substantial negative impact of HS during the reproductive phase of plants, reinforcing the importance of investigating genes that may enhance tolerance to such conditions.

Therefore, it would be important to continue the work developed in this thesis through the following approaches:

- Analysis of the expression levels of *OsERF98* and *OsBIP2* via RT-qPCR;
- Characterization of the mutant lines generated using the constructs developed in this study, in both *Arabidopsis* and rice, focusing on reproductive phenotypes associated with HS tolerance;
- Obtention and phenotypic analysis of new T-DNA insertion lines or using CRISPR-Cas9 to obtain knock-out mutant lines for *AtERF114* and *AtYAB3*, given that the analyzed line was not a true knock-out and thus did not allow definitive conclusions to be drawn regarding the function of the gene under investigation;
- Repetition of the HS assays to confirm the robustness of the obtained results.

Finally, although only a subset of the genes identified through the previous RNA-seq analysis was selected for investigation in this study, the remaining genes should not be overlooked. It is essential to continue analysing these candidate genes, as they may also play important roles in the complex mechanisms underlying plant tolerance to HS.

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Attachments

Table 22. Bibliographic analysis of the genes overexpressed in HS conditions in N22.

Name	ID_LOC	RAP_DB	RPKM (C)	RPKM (HS)	FC (HS/C)	log2FC	FDR	Expression tissues	subcellular localization	Molecular function (GO)	Trait ontology	Downregulated mutant phenotype	Overexpression mutant phenotype
<i>GELF87</i>	LOC_Os09g043044	Os09g0436700	6.22	15.55	2.50	1.32	3.10E-02	Anther, stem maturation; inflorescence; ovary leaf; lemma; palea		Hydrolase of ester bonds	Heat tolerance		
<i>GELP12</i>	LOC_Os01g012320	Os01g0223200	10.39	24.28	2.34	1.22	2.45E-03			Hydrolase of ester bonds	Heat tolerance		
<i>GELP46</i>	LOC_Os02g057110	Os02g0816200	25.9	58.09	2.24	1.17	1.67E-07			Hydrolase of ester bonds			
<i>OscER1</i>	LOC_Os02g040784	Os02g0821300	8.85	18.66	2.11	01.08	4.17E-02	Anther; Tapetum/bicellular pollen grains	Endoplasmatic reticulum	biosynthesis of VLC alkane, regulation of anther and pollen development, plastid differentiation; distinct vas. synthesis pathway; iron ion binding, oxidoreductase activity		sterile; fewer pollen antheriploids; delay in PCO, plastid defects; fewer alkanes; more waxes; alkenes and primary alcohols	Increased concentration of C27 alkanes
<i>GPAT</i>	LOC_Os03g002340	Os03g0114400	3.7	10.76	2.91	1.54	4.40E-02	roots and anthers	plasmatic membrane	Synthesis of the polyester extracellular lipid barrier			
<i>OsRS5</i>	LOC_Os01g07530	Os01g0710000	13.35	36.87	2.76	1.47	1.12E-06	roots, anthers and seeds		Carbohydrate metabolic process; response to oxidative stress; response to drought stress	peticid sensitivity		
<i>ERF38</i>	LOC_Os03g34280	Os02g0546800	0.16	29.82	186.38	7.54	7.40E-03	ovary		Transcription factor, defense-activated signaling pathway			
<i>OsLEA17</i>	LOC_Os03g00680	Os03g0329000	21.06	57.2	2.72	1.44	3.27E-10	Anther	nucleus	Sensitivity to ABA; tolerance to salinity; heat tolerance; thermotolerance in pollen tubes, ATP-binding, ATP-dependent chaperone		better growth in high salinity; recovers after 20 days under drought stress	
<i>OsBIP2</i>	LOC_Os03g050250	Os03g0710500	4.29	76.8	17.9	4.16	0.00E+00	Anther	nucleus; cytoplasm; RE; membrane	heat tolerance; thermotolerance in pollen tubes, ATP-binding, ATP-dependent chaperone			
<i>OsYABBY7</i>	LOC_Os07g038410	Os07g0571800	4.3	15.05	3.5	1.81	1.40E-03	psill		Meiosis-specific DNA recombinase; SC assembly; DNA metabolic process and repair; recombination	masculine sterility	defects in homologue pairing	
<i>DMC1B</i>	LOC_Os11g04954	Os11g0146800	3.4	20.87	6.14	2.62	1.20E-07	anther, pollen mother cells; stem and root tips	Nucleus				
<i>ZEP1</i>	LOC_Os04g077960	Os04g0452500	1.92	9.31	4.85	2.28	6.53E-03	anther, ovary	Nucleus	regulation of the number of crossovers during meiosis; DNA metabolic process; chiasma termination; protein binding		Does not assemble SC; crossovers are well formed, but the SC is not properly assembled; chiasma terminalization does not occur; <i>PAIR2</i> and <i>MER3</i> dissociation is delayed; defects in pollen development; early pollen condensation; preventing polar migration and leading to pollen abortion	
<i>G47</i>	LOC_Os07g03940	Os07g0033100	6.49	23.06	3.55	1.83	1.32E-05	roots; stem; inflorescence; anther		actin depolymerization; abiotic stress response; induced by osmotic stress and drought	Germination rate		
<i>OsADP3</i>	LOC_Os03g060580	Os03g0205000	26.98	76.8	2.85	1.51	5.74E-03	vascular tissues; lemma; ovary; embryo; endosperm	Nucleus		Cold tolerance		
<i>TIP1-2</i>	LOC_Os01g04450	Os01g0575900	7.25	21.31	2.94	1.56	8.93E-04	leaf	membranes	Aquaporin; arsenite tolerance; transmembrane transport	salinity and cold tolerance		
<i>TIP1-1</i>	LOC_Os03g05290	Os03g0146100	193.69	518.14	2.68	1.42	0.00E+00	leaf; roots; stem; palea and lemma	membranes	Aquaporin; transmembrane transport	salinity and cold tolerance		
<i>TIP2-2</i>	LOC_Os06g022940	Os06g0336200	170.85	363.54	2.13	1.09	0.00E+00	leaf; roots; stem; embryo	membranes	Aquaporin; transmembrane transport	salinity and cold tolerance; resistance to bacterial diseases; tolerance to salinity; drought and cold; sensitivity to ABA and boron		
<i>PIP2-6</i>	LOC_Os04g16450	Os04g0233400	42.8	90.91	2.12	1.09	1.35E-10	roots and shoots; vascular tissues; leaf; stem; palea and lemma	membranes	Aquaporin; transmembrane transport; arsenic translocation from roots to shoots	reduction of AS accumulation in roots; shoots, leaves and grains	Increased arsenic accumulation in shoots	
<i>NP2-2 (LS6)</i>	LOC_Os06g12310	Os06g0228200	12.97	26.46	2.04	1.03	9.04E-03	xylem; root tips	membranes	aquaporin; transport of silicon out of the xylem, affecting its distribution in the leaves; accumulation of silica helps plants cope with biotic and abiotic stress	disordered deposition of silica in the shoots; increased excretion of silicon by graminaceous plants; accumulation of Si in the panicles and increased accumulation in th. glab leaf		
<i>RSUS4</i>	LOC_Os03g22120	Os03g0405000	83.47	190.58	2.28	1.19	5.74E-08	leaf, mature seeds; endosperm		sucrose metabolism; glycotransferase activity; sucrose synthase activity	nitrogen sensitivity; seed weight; number of grains per plant; salinity and drought tolerance; starch content	increased fertility	
<i>OsUGT1</i>	LOC_Os05g51670	Os05g0595100	117.92	250.33	2.19	1.13	0.00E+00	anther; tapetum and pollen grains; SA macrospores; endosperm	cytoplasm; nucleus	sugar metabolism; galactose metabolism; UDP-glucose-4-epimerase activity; regulates UDP-sugar homeostasis and pollen sterility; interconversion of UDP-Glc and UDP-Gal; controls anther development; regulates UDP-sugar homeostasis; ROS accumulation; anther wall PCO; callose degradation; pollen wall development	drought tolerance	Leaves accumulated more glucose and galactose in hemicellulose under low sucrose conditions; accumulation of less cellulose in shoot-stained cell wall C-partitioning under N-limited conditions via repression of genes associated with cellulose synthesis mediated by UGE1; coleoptiles grew more in the absence of water stress; increased tolerance to salinity and tolerance to oxidative stress; increased glc and decreased xy in leaves under N limitation	

Table 23. List of primers used during this study.

Primers	Sequence (5'-3')	Amplificate
1765	GGGGACAAGTTTGTACAAAAAAGCAGGCT ATGTATGGGAAGAGGCCTTTTG	<i>AtERF114</i> (CDS)
1766	GGGGACCACTTTGTACAAGAAAGCTGGGT TTAATATCCCGAATGAGGAGAG	
1767	GGGGACAAGTTTGTACAAAAAAGCAGGCT ATGTCGAGCATGTCCATGTC	<i>AtYAB3</i> (CDS)
1768	GGGGACCACTTTGTACAAGAAAGCTGGGT CTAGTTATGGGCCACCCC	
1769	GGGGACAAGTTTGTACAAAAAAGCAGGCT TGAACACGTGAACAAATGGCT	<i>pERF114</i>
1770	GGGGACCACTTTGTACAAGAAAGCTGGGT TCCGAGGATCTACTTTTGCTTGA	
1771	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCAACTTCGACGCCTTGTGT	<i>pYAB3</i>
1772	GGGGACCACTTTGTACAAGAAAGCTGGGT AGAGAGAGAGGAGGGCTTTGA	
1777	CTAGCTAAACCTATAGATGC	<i>AtYAB3</i> (Sequencing)
1779	ACTATATATTGTGAAACCGG	<i>pYAB3</i> (Sequencing)
1803	CCCTAGATCGTAGATCCTAAGGC	<i>yab3</i> (genotyping)
1804	CATCTACAAGAGATGCCTCGG	
LBb3.1	ATTTTGCCGATTTCCGAAC	
250	CTGTTACGGAACCCAATTC	<i>AtRUB1</i>
251	GGAAAAAGGTCTGACCGACA	
252	CTCAGGTATTGCAGACCGTATGAG	<i>AtACT8</i>
253	CTGGACCTGCTTCATCACTCTG	
1801	GAGGGTTCAGCTTGAAGTAATC	<i>AtERF114</i>
1802	GAATATCCGGTACCACTCCCATAC	
1799	GAGCATTTGGTTATGGCGACTC	<i>AtYAB3</i>
1800	CCTGCCTTTATACGTTGGATCTCC	
1682	GAGCCTCTGTTCTGCAAGTA	<i>OsUBQ</i>
1683	ACTCGATGGTCCATTAAACC	
1684	TGGTATGGTGGTGACCTTTG	<i>OsEF1</i>
1685	GTACCCACGCTTCAGATCCT	
1814	GTGTTGCTGGTGAGCGTG	<i>OsYABBY7</i>
1815	CATACCTGTGGAGTCAGCTC	
29	ATTTCTTGAGATCCGAATTCGGTACCATGCCCTCAGCAAGATCAAGTG	<i>OsERF98</i> (CDS)
30	AGAAGCTTCCATGGGTGCACTCAAGTTCTGTGGACTAGGAGTA	
31	ATTTCTTGAGATCCGAATTCGGTACCTCACCAAATCGCACATCGTAG	<i>OsBIP2</i> (CDS)
32	AGAAGCTTCCATGGGTGCACTGGAGCAGCGAAAATGCTCTA	
33	GCAGGCAGTGAGCTGAGAGAGTAA	<i>pegYABBY7</i>
34	AACTTACTCTCTCAGCTGCACTGC	
35	GTGCTTTTCCATTAATTCTAGAATCTCTCAGCCTTTAAAC	<i>extYABBY7</i>
36	CGCGGTTTAAAGGCTGAGAGAGATTCTAGAATTAATGGAAAAA	
50	CAATTAGCCCCGGAGATATC	<i>pegHSP-R</i>

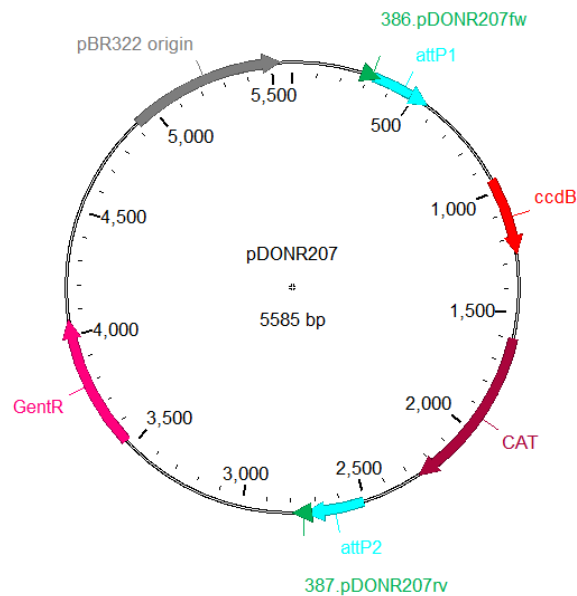


Fig. 32. Schematic representation of the Gateway entry vector *pDONR207*. The *ccdB* gene is a lethal gene used for negative selection, allowing for the identification of bacterial colonies that do or do not contain the insert. The *ccdB* gene is toxic for most *E. coli* strains, such as DH5 α , with the exception of the DB3.1 strain, which carries a mutation that confers resistance to *ccdB*-mediated toxicity. The binding sites for the primers pDONR207fw and pDONR207rv are also represented. GentR - gentamicin resistance gene.

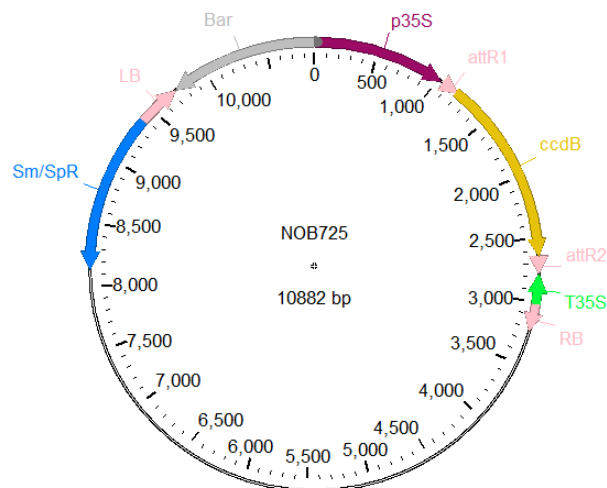


Fig. 33. Schematic representation of the Gateway expression vector *NOB725* (*pB2GW7*). *p35S* - constitutive promoter 35S; Bar - Basta resistance gene; SpR - spectinomycin resistance gene.

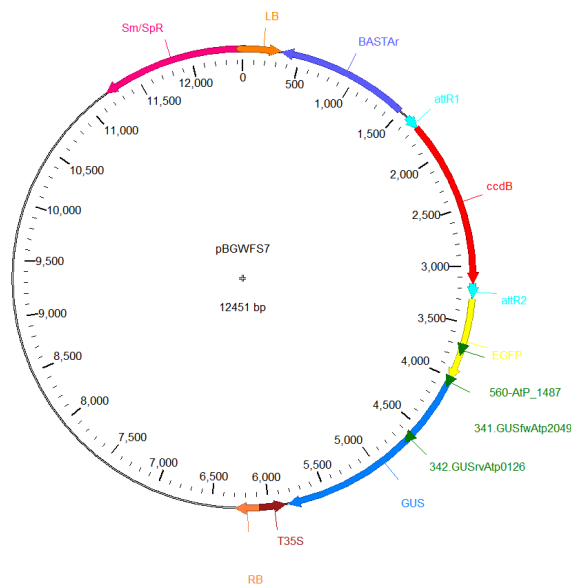


Fig. 34. Schematic representation of the Gateway expression vector *NOB724.1* (*pBGWFS7*). *GUS* - β -glucuronidase coding sequence; Bar - Basta resistance gene; SpR - spectinomycin resistance gene.

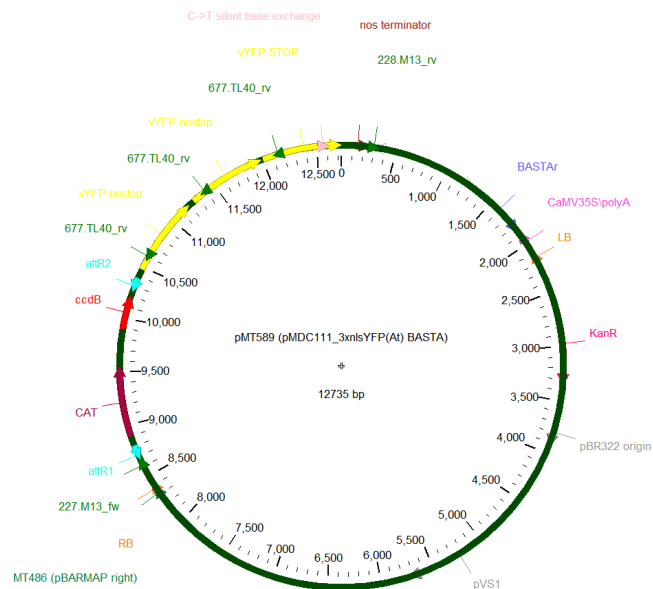


Fig. 35. Schematic representation of the Gateway expression vector *pMT589*. *YFP* - yellow fluorescent protein coding sequence; Bar - Basta resistance gene; KanR - kanamycin resistance gene.

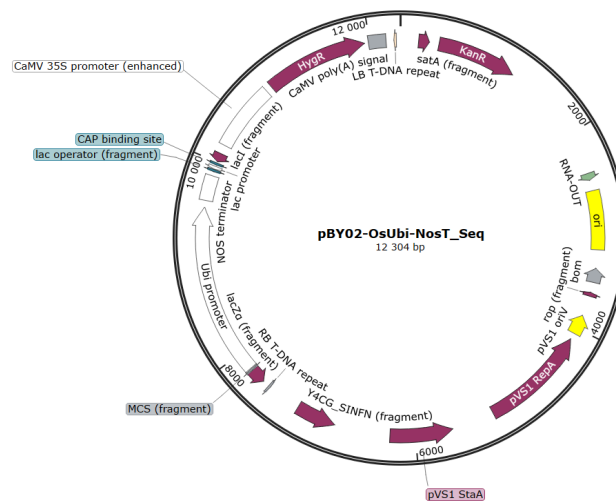


Fig. 36. Schematic representation of the *pBY02* plasmid. KanR - kanamycin resistance gene.

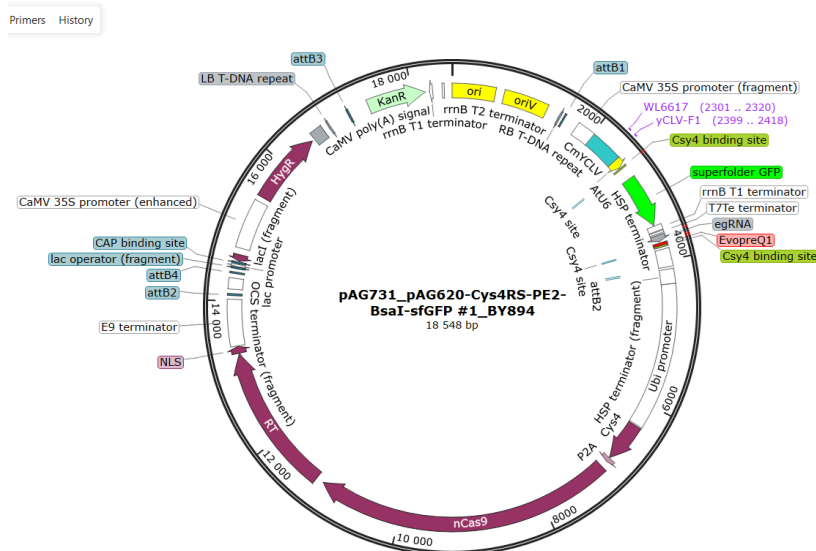


Fig. 37. Schematic representation of the *pAG731* plasmid. *superfolder GFP* - green fluorescent protein coding sequence, for selection of the negative colonies. During the Golden gate reaction, this sequence is substituted for the interest sequence; KanR - kanamycin resistance gene.

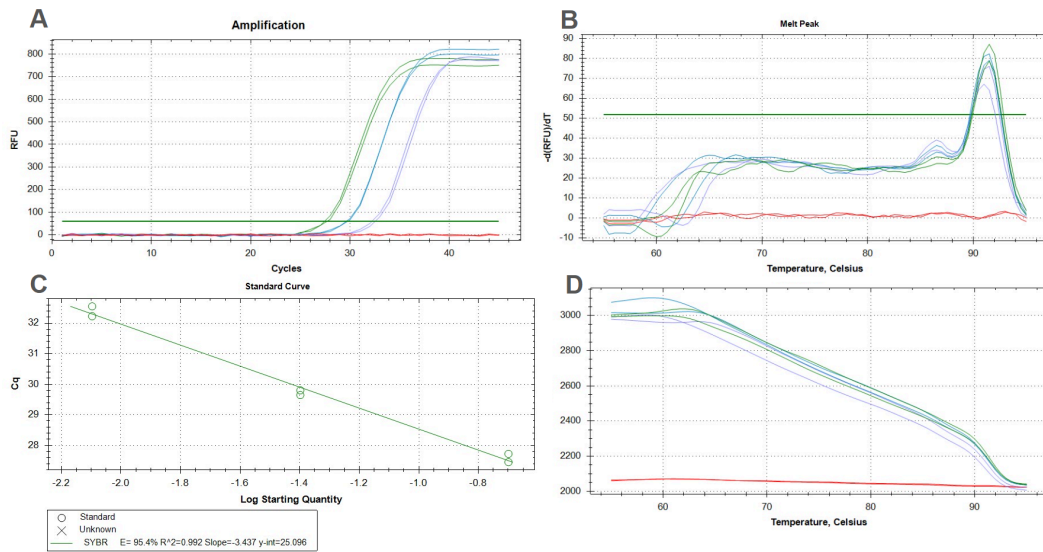


Fig. 38. Efficiency of RT-qPCR *OsYABBY7* primers in *O. sativa* inflorescences. **A** - Amplification curve for *OsYABBY7* primers, showing a consistent and specific amplification across dilution series (green: 1:5; blue: 1:25; purple: 1:125; red: NTC). **B** - Melt peak analysis for *OsYABBY7* primers, showing only one melt peak corresponding to the specific amplification for this gene. **C** - Standard curve for *OsYABBY7* primers, showing high efficiency (95.4%) and linearity ($R^2 = 0.992$). **D** - Melt curve analysis for *OsYABBY7* primers.

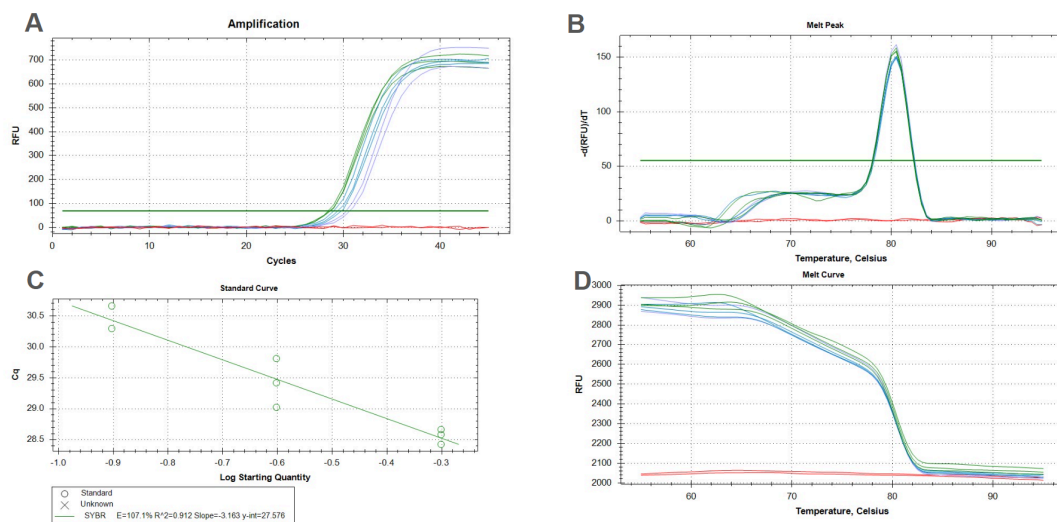


Fig. 39. Efficiency of RT-qPCR *AtERF114* primers in *A. thaliana* inflorescences. **A** - Amplification curve for *AtERF114* primers, showing a consistent and specific amplification across dilution series (green: 1:2; blue: 1:4; purple: 1:8; red: NTC). **B** - Melt peak analysis for *AtERF114* primers, showing only one melt peak corresponding to the specific amplification for this gene. **C** - Standard curve for *AtERF114* primers, showing high efficiency (107.1%) and linearity ($R^2 = 0.912$). **D** - Melt curve analysis for *AtERF114* primers.

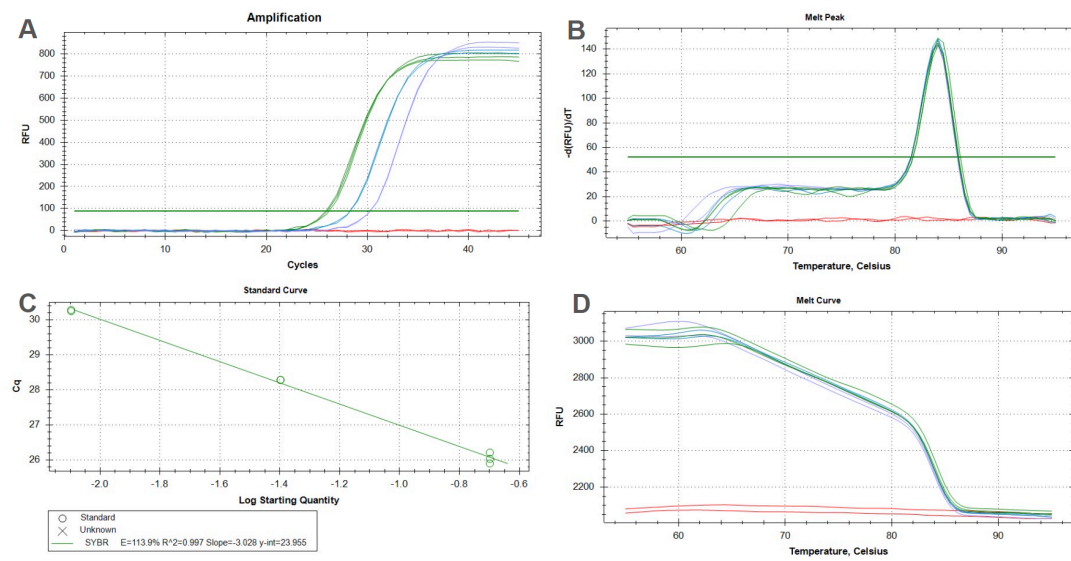


Fig. 40. Efficiency of RT-qPCR *AtYAB3* primers in *A. thaliana* inflorescences. **A** - Amplification curve for *AtYAB3* primers, showing a consistent and specific amplification across dilution series (green: 1:5; blue: 1:25; purple: 1:125; red: NTC). **B** - Melt peak analysis for *AtYAB3* primers, showing only one melt peak corresponding to the specific amplification for this gene. **C** - Standard curve for *AtYAB3* primers, showing high efficiency (113.9%) and linearity ($R^2 = 0.997$). **D** - Melt curve analysis for *AtYAB3* primers.

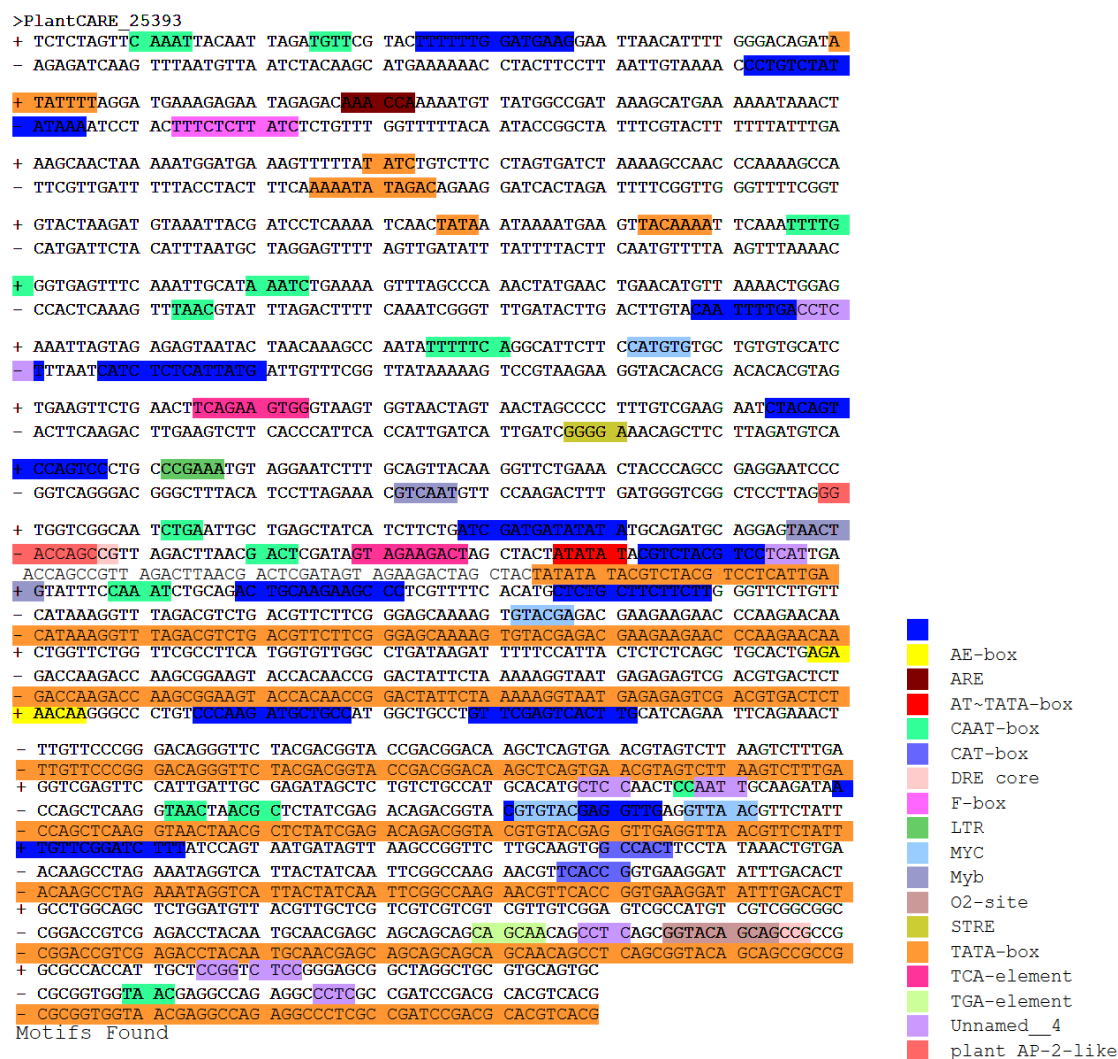


Fig. 41. OsYABBY7 promoter analysis, using the PlantCare software.

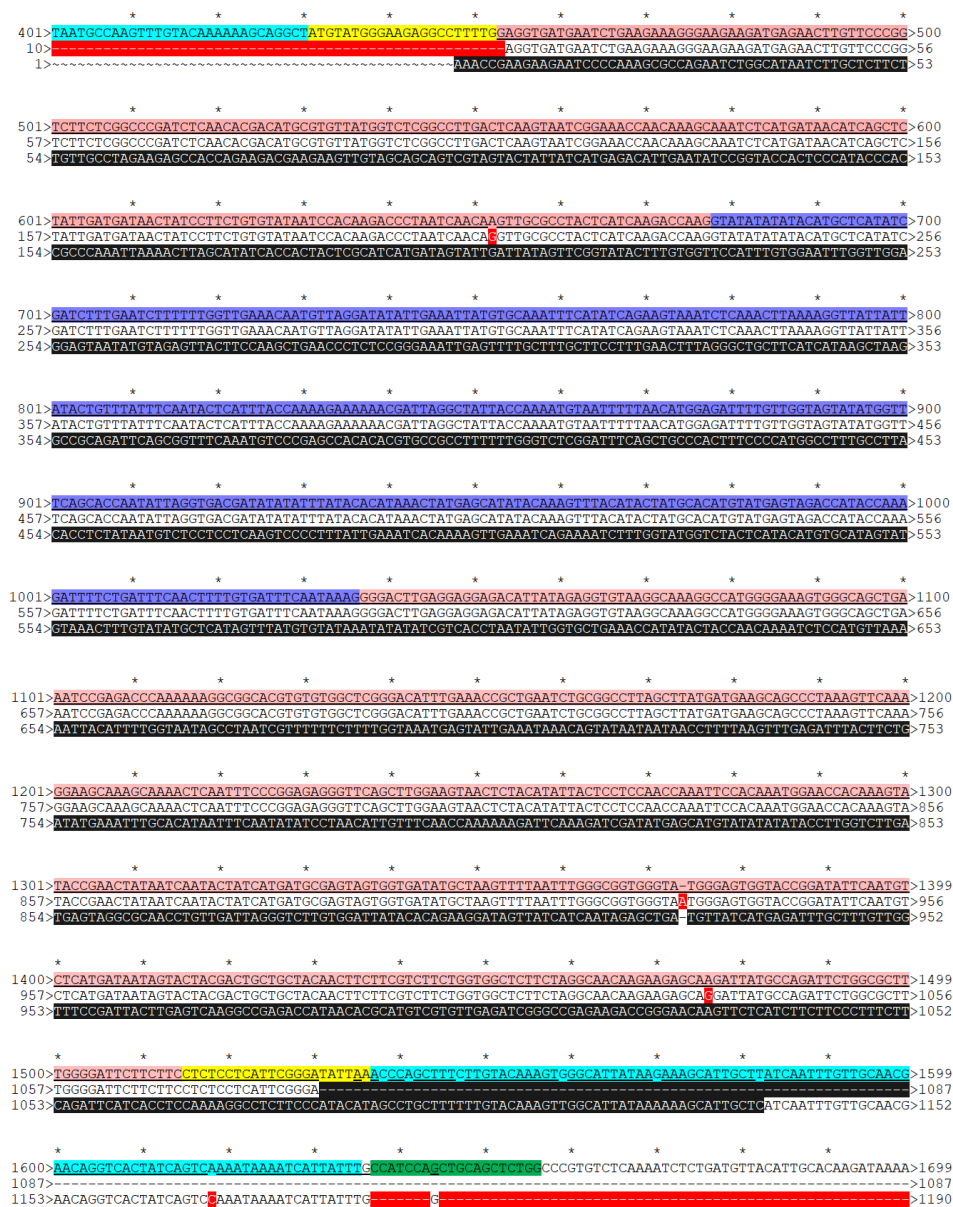


Fig. 42. Alignment results between the *pDONR207::ERF114* expected sequence and the sequencing result of the DNA fragment amplified with the 1765 and 1766 primer pair.



Fig. 43. Alignment results between the *pDONR207::pERF114* expected sequence and the sequencing result of the DNA fragment amplified with the 1769 and 1770 primer pair.

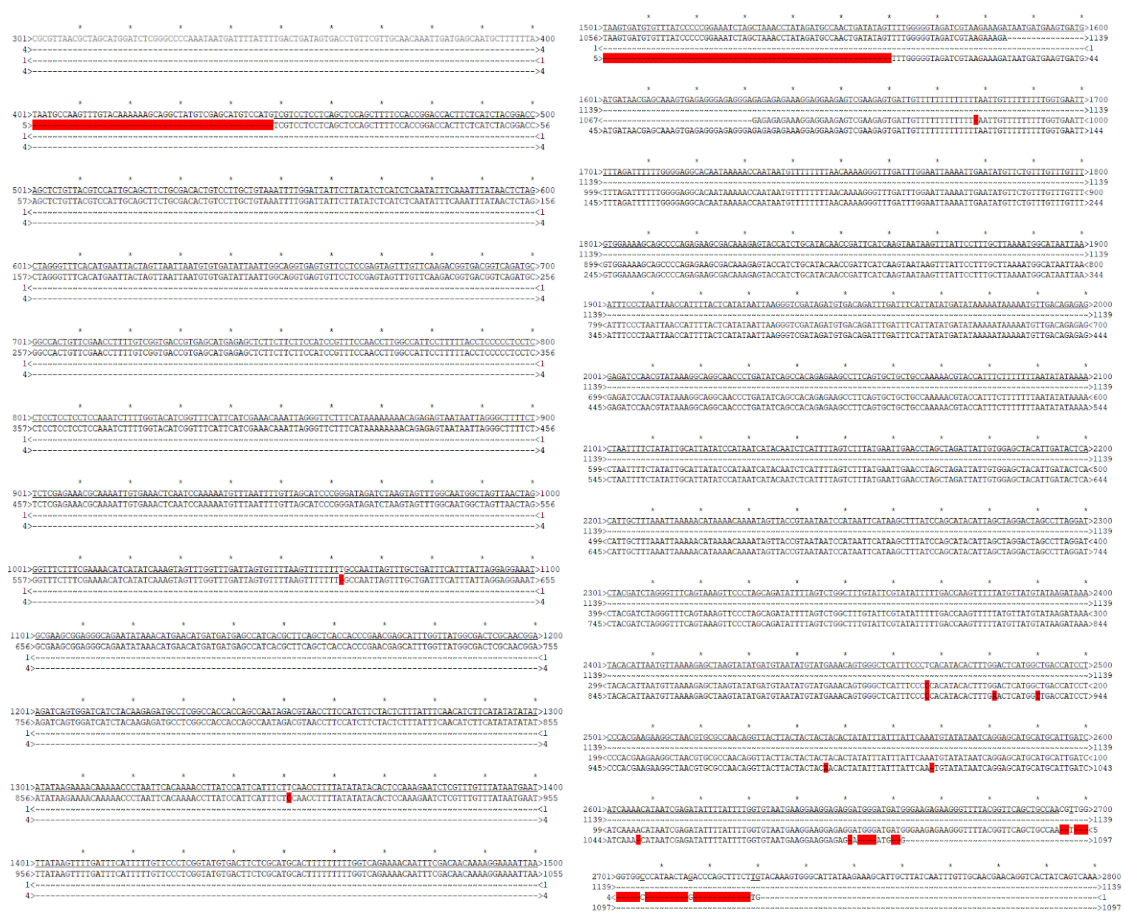


Fig. 44. Alignment results between the *pDONR207::YAB3* expected sequence and the sequencing result of the DNA fragment amplified with the 1767, 1768 and 1777 primers.



Fig. 46. Alignment results between the *pB2GW7::ERF114* expected sequence and the sequencing result of the DNA fragment amplified with the 1765 and 1766 primer pair.

1501>ccggggtacctctagatgcacctgcaggcatcgcaagtctcccocatgtgtgcacgtcgaagcgccgcactagtgatataccaagtttGTGACAAAAGAA-1700
4>----->4
1079<-----<1079

1601>CAGSCTTGAANAAGTGAAACAATAATGGCTATTGATCACACTATAAATTTTCGTCAAATAAATGAATAGTAGTAGTACACATGTGATCTAAGATTIAGATGA-1700
5>-----TTGATCACACTATAAATTTCCGTCAAATAAATGAATAGTAGTAGTACACATGTGATCTAAGATTIAGATGA-76
1079<-----<1079

1701>GGGTGCCAAGTGAAGACCABAATACATTTAACCACTAAGCTACAGTTTAGAGAATTTTGATCATAAACATAAATAAACACCTGGTTAGGGAAGTGGTA-1800
77>GGGTGCCAAGTGAAGACCABAATACATTTAACCACTAAGCTACAGTTTAGAGAATTTTGATCATAAACATAAATAAACACCTGGTTAGGGAAGTGGTA-176
1079<-----<1079

1801>GTTGAACCTAACACCAACGACGAGTACACATGATCGATCAAGTTAGTCAAACTGGCTGTGGAITACATTTAAGCTCTTACATATTATATCCGTAAGTCT-1900
177>GTTGAACCTAACACCAACGAGTACACATGATCGATCAAGTTAGTCAAACTGGCTGTGGAITACATTTAAGCTCTTACATATTATATCCGTAAGTCT-176
1079<-----<1079

1901>TCACACATGTGATACATAATTTCTTACCTGTCAACTTAGTTAGCTCTTGACCTTTTTTGAGTGAATAATTTTCAAATGTCAAATTTTTCATACACAAGCT-2000
297>TCACACATGTGATACATAATTTCTTACCTGTCAACTTAGTTAGCTCTTGACCTTTTTTGAGTGAATAATTTTCAAATGTCAAATTTTTCATACACAAGCT-376
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2001>ATAATATGTAGATAGGCAGAGCTCCATTGAGTAAAAATTAATACCTCCTTTTCGACGTAGAAAGCTGTACACACCCCTAACACTTGCATTTTAAACAA-2100
77>ATAATATGTAGATAGGCAGAGCTCCATTGAGTAAAAATTAATACCTCCTTTTCGACGTAGAAAGCTGTACACACCCCTAACACTTGCATTTTAAACAA-476
1079<-----AACCTAACACTTGCATTTTAAACAA-1054

2101>TTTCTACAAATGTACACCTAACCAATTAATCAATTCATCATACATAGACATAGAACATAATCACTTTTGTTAACTCAATTCGAGTGTGTACACGATA-2200
1053>TTTCTACAAATGTACACCTAACCAATTAATCAATTCATCATATAGACATAGAACATAATCACTTTTGTTAACTCAATTCGAGTGTGTACACGATA-954

2201>CTTGGATTAGAGGTTTTTAAAGGCTTTTTGTGTGCAATTT-TAGTCAACGAGCTTTAAATAAATTAATAGGTTCACTCTTACGTACGCAAACTC-2299
577>CTTGGATTAGAGGTTTTTAAAGGCTTTTTGTGTGCAATTT-TAGTCAACGAGCTTTAAATAAATTAATAGGTTCACTCTTACGTACGCAAACTC-675
953>CTTGGATTAGAGGTTTTTAAAGGCTTTTTGTGTGCAATTT-TAGTCAACGAGCTTTAAATAAATTAATAGGTTCACTCTTACGTACGCAAACTC-855

2300>ATAATTCCTTTTAAATTCACGTTTGGATCCACCAAGGGGTGTGGTTTACATATATATATAGATTTTCTTAATACCTTTTCTTGAGAATGCAGGTGGA-2399
476>ATAATTCCTTTTAAATTCACGTTTGGATCCACCAAGGGGTGTGGTTTACATATATATATAGATTTTCTTAATACCTTTTCTTGAGAATGCAGGTGGA-775
854>ATAATTCCTTTTAAATTCACGTTTGGATCCACCAAGGGGTGTGGTTTACATATATATATAGATTTTCTTAATACCTTTTCTTGAGAATGCAGGTGGA-755

2400>TTTTTGACGAAAAAACAATGTGTGGTTTAAATTCATATACACTAAGACCACGACAGTCTTTTATAGTTTAAATTTATATTTAGACAAAAAACAC-2499
776>TTTTTGACGAAAAAACAATGTGTGGTTTAAATTCATATACACTAAGACCACGACAGTCTTTTATAGTTTAAATTTATATTTAGACAAAAAACAC-875
754>TTTTTGACGAAAAAACAATGTGTGGTTTAAATTCATATACACTAAGACCACGACAGTCTTTTATAGTTTAAATTTATATTTAGACAAAAAACAC-655

2500>TATTAGTCTATATCTTCCCCTCAATTCATGAATGTGTAAACCTTTTCACACAAAATAAATGATTAACAAAACATGACATGATGCGCAATTTTCC-2599
876>TATTAGTCTATATCTTCCCCTCAATTCATGAATGTGTAAACCTTTTCACACAAAATAAATGATTAACAAAACATGACATGATGCGCAATTTTCC-975
654>TATTAGTCTATATCTTCCCCTCAATTCATGAATGTGTAAACCTTTTCACACAAAATAAATGATTAACAAAACATGACATGATGCGCAATTTTCC-555

2600>TCTCTCTTTTATTTTATCTTTTACCTTTTGCATGTTTGTAGTTTTCCTCATCTCGTGATGATTTTATAGTAACTTTTATAAAATATCTCTCTTTTGATAT-2699
976>TCTCTCTTTTATTTTATCTTTTACCTTTTGCATGTTTGTAGTTTTCCTCATCTCGTGATGATTTTATAGTAACTTTTATAAAATATCTCTCTTTTGATAT-107
554>TCTCTCTTTTATTTTATCTTTTACCTTTTGCATGTTTGTAGTTTTCCTCATCTCGTGATGATTTTATAGTAACTTTTATAAAATATCTCTCTTTTGATAT-455

2700>TTAAATACACATGCACCTTCTCTCAATGAAGAAAGTTTAAACAACTTCTCATATACAGCTTCTTACATACAAACATGATCGGATCTGGTGTACAGG-2799
1073>TTAAATACACATGCACCTTCTCTCAATGAAGAAAGTTTAAACAACTTCTCATATACAGCTTCTTACATACAAACATGATCGGATCTGGTGTACAGG-2799
454>TTAAATACACATGCACCTTCTCTCAATGAAGAAAGTTTAAACAACTTCTCATATACAGCTTCTTACATACAAACATGATCGGATCTGGTGTACAGG-355

2800>ATATTTTAAATACGATACAGAAACAAACATATAGAAATTTTAAATACGAAAGATGTGAAAAAATAAATAAATCAAACTTAAATACATTT-2899
1164>ATATTTTAAATACGATACAGAAACAAACATATAGAAATTTTAAATACGAAAGATGTGAAAAAATAAATAAATCAAACTTAAATACATTT-1164
354>ATATTTTAAATACGATACAGAAACAAACATATAGAAATTTTAAATACGAAAGATGTGAAAAAATAAATAAATCAAACTTAAATACATTT-255

2900>GTTTAGGCTCATATGTTACACGTAAGCAAGTCTCTCTATAAAGGCTCAGAAAATTGCTAAACCCCCCAAAAAATACTTTACTCTCTCTTTCTTCA-2999
1167>GTTTAGGCTCATATGTTACACGTAAGCAAGTCTCTCTATAAAGGCTCAGAAAATTGCTAAACCCCCCAAAAAATACTTTACTCTCTCTTTCTTCA-1167
254>GTTTAGGCTCATATGTTACACGTAAGCAAGTCTCTCTATAAAGGCTCAGAAAATTGCTAAACCCCCCAAAAAATACTTTACTCTCTCTTTCTTCA-155

3000>TTTACTCAATCTAAATTTCTCTCTATCATCTCTTGTTCTTGATGTTCAACGATGCCATAAAGAAAACTGAACACCCCTAAGTACTGTATAAATC-3099
1167>TTTACTCAATCTAAATTTCTCTCTATCATCTCTTGTTCTTGATGTTCAACGATGCCATAAAGAAAACTGAACACCCCTAAGTACTGTATAAATC-1167
154>TTTACTCAATCTAAATTTCTCTCTATCATCTCTTGTTCTTGATGTTCAACGATGCCATAAAGAAAACTGAACACCCCTAAGTACTGTATAAATC-55

3100>CTATATCAAAATTTTTTTTTGGAAGAAAGGCTATATTTAAAGAAATCAAGCAAAAGTAGATCTCGGAAACCGAGCTTTCTgtgtacaaagtggtgata-3199
1167>CTATATCAAAATTTTTTTTTGGAAGAAAGGCTATATTTAAAGAAATCAAGCAAAAGTAGATCTCGGAAACCGAGCTTTCTgtgtacaaagtggtgata-1167
54>CTATATCAAAATTTTTTTTTGGAAGAAAGGCTATATTTAAAGAAATCAAGCAAAAGTAGATCTCGGAAACCGAGCTTTCTgtgtacaaagtggtgata-54

Fig. 47. Alignment results between the *pBGWFS7::pERF114* expected sequence and the sequencing result of the DNA fragment amplified with the 1769 and 1770 primer pair.

```

8601>ggttttccagtcacgacgttgtaaaacgacggccagtgccaagcttagttaattaagaattatcaaacagttgtacaaaaagcaggcttgaaaacg>8700
4>----->4
1067<-----<1067

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
8701>TGAACAAATGGCTATTGATCAGACTATAAAATTTCCGTCAAATAAATGAATAGTTAGTATCAGATGATCTAAGATTAGATGAGGGTGTCCAAAGT>8800
5>-----ATTGATCAGACTATAAAATTTCCGTCAAATAAATGAATAGTTAGTATCAGATGATCTAAGATTAGATGAGGGTGTCCAAAGT>91
1067<-----<1067

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
8801>AAGACCAATACATTTAAGCTAAGCTACAGTTTAGAGAAATTTTATCATAAACAATAAACAAGCTGGTTAGGGAAGTGGTATTGAACCTAACAC>8900
192>AAGACCAATACATTTAAGCTAAGCTACAGTTTAGAGAAATTTTATCATAAACAATAAACAAGCTGGTTAGGGAAGTGGTATTGAACCTAACAC>191
1067<-----<1067

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
8901>CAACGAGTACACATGATGATCAAGTTAGTCAAACTGGCTGGGATTACATTACAGCTTTACATTATTATATCGTACTTGTGCACAGATTGTACA>9000
192>CAACGAGTACACATGATGATCAAGTTAGTCAAACTGGCTGGGATTACATTACAGCTTTACATTATTATATCGTACTTGTGCACAGATTGTACA>291
1067<-----<1067

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
9001>TAATTTCTTTACCTGTCACTTAGTATGCTCTTGACTTTTTTGAGTGAATAATTTTCAATGTCAAAATTTTATACATCAAGCCTATAATATGTAGATA>9100
292>TAATTTCTTTACCTGTCACTTAGTATGCTCTTGACTTTTTTGAGTGAATAATTTTCAATGTCAAAATTTTATACATCAAGCCTATAATATGTAGATA>391
1067<-----<1067

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
9101>GGCAAGAGCTCCATTGAGTAAAAATTAATACCTCTTTTCGGACGTAGAAACGTGTACACACCTTAACACTTGCATTTTAAACAATTTTACAAAATGT>9200
392>GGCAAGAGCTCCATTGAGTAAAAATTAATACCTCTTTTCGGACGTAGAAACGTGTACACACCTTAACACTTGCATTTTAAACAATTTTACAAAATGT>491
1066>-----TGCATTTTAAACAATTTTACAAAATGT<1039

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
9201>AACACTAACCTTTAATCAATTTTATCATATACAGCATAGAACAAATATCACTTTGTTTAACTCAATTCGAGTGTGTACACGATCTTGGATTAGAGG>9300
492>AACACTAACCTTTAATCAATTTTATCATATACAGCATAGAACAAATATCACTTTGTTTAACTCAATTCGAGTGTGTACACGATCTTGGATTAGAGG>591
1038>AACACTAACCTTTAATCAATTTTATCATATCTTGCATAGAACAAATATCACTTTGTTTAACTCAATTCGAGTGTGTACACGATCTTGGATTAGAGG>939

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
9301>TTTTTAAAGCTCTTTTGTGTGCAAAATTTTATGCAACAGCTCTTTAAATAAATTAATAGGTTCACTCTTACGTACGCAAACTCATATTTCTTTTAA>9400
592>TTTTTAAAGCTCTTTTGTGTGCAAAATTTTATGCAACAGCTCTTTAAATAAATTAATAGGTTCACTCTTACGTACGCAAACTCATATTTCTTTTAA>9400
938>TTTTTAAAGCTCTTTTGTGTGCAAAATTTTATGCAACAGCTCTTTAAATAAATTAATAGGTTCACTCTTACGTACGCAAACTCATATTTCTTTTAA>840

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
9401>TTACAGTTTGGATCCACAAAGGGGTGCGGTTTACATTATATATAGATTCTTAAATACCTTTTCTTGAGATGCAAGTGTGATTTTGACGAAAAAA>9500
692>TTACAGTTTGGATCCACAAAGGGGTGCGGTTTACATTATATATAGATTCTTAAATACCTTTTCTTGAGATGCAAGTGTGATTTTGACGAAAAAA>791
839>TTACAGTTTGGATCCACAAAGGGGTGCGGTTTACATTATATATAGATTCTTAAATACCTTTTCTTGAGATGCAAGTGTGATTTTGACGAAAAAA>740

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
9501>ACAATGTGCGGTTTAAATTTACATATACACTAAGACACGACAGTCTTTTATAGTTTAAATTTAATTTAGCAAAAAACACTATTAGCTATATCT>9600
792>ACAATGTGCGGTTTAAATTTACATATACACTAAGACACGACAGTCTTTTATAGTTTAAATTTAATTTAGCAAAAAACACTATTAGCTATATCT>891
739>ACAATGTGCGGTTTAAATTTACATATACACTAAGACACGACAGTCTTTTATAGTTTAAATTTAATTTAGCAAAAAACACTATTAGCTATATCT>640

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
9601>TTCCCTCAATTCATGAATGTGTAAACCCCTTTTACACAAAATAAATGATTAAACAAAACATGAAATGCAATTTTCTCTCTCTTTATTTT>9700
892>TTCCCTCAATTCATGAATGTGTAAACCCCTTTTACACAAAATAAATGATTAAACAAAACATGAAATGCAATTTTCTCTCTCTTTATTTT>990
639>TTCCCTCAATTCATGAATGTGTAAACCCCTTTTACACAAAATAAATGATTAAACAAAACATGAAATGCAATTTTCTCTCTCTTTATTTT>540

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
9701>ATTCCTTTTACCTTTTCAATGTTTATTTTCCATCTCGTGTAGTATTTATAGTAATCTTTTAAATATCTCTCTTTTATATTTAATACACATGCA>9800
991>ATTCCTTTTACCTTTTCAATGTTTATTTTCCATCTCGTGTAGTATTTATAGTAATCTTTTAAATATCTCTCTTTTATATTTAATACACATGCA>1088
539>ATTCCTTTTACCTTTTCAATGTTTATTTTCCATCTCGTGTAGTATTTATAGTAATCTTTTAAATATCTCTCTTTTATATTTAATACACATGCA>440

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
9801>CTTTCTCAATAAGAAAGTTTAAACAACTTTCATTATACATGCTTCAATTACAAAACATGATCGGTACTGGTGTACAGCATATTTTAAATATC>9900
1089>CTTTCTCAATAAGAAAGTTTAAACAACTTTCATTATACATGCTTCAATTACAAAACATGATCGGTACTGGTGTACAGCATATTTTAAATATC>1124
439>CTTTCTCAATAAGAAAGTTTAAACAACTTTCATTATACATGCTTCAATTACAAAACATGATCGGTACTGGTGTACAGCATATTTTAAATATC>340

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
9901>GATAACAGAAACAAACATATATAGAAATTTTAAATACGAAAGATGTGAAAAAAGAAAAAATCAAACTTAAACATATTTGTTTATAGTCATATG>10000
1124>----->1124
339>GATAACAGAAACAAACATATATAGAAATTTTAAATACGAAAGATGTGAAAAAAGAAAAAATCAAACTTAAACATATTTGTTTATAGTCATATG>240

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
10001>TTACAGCTAGAACGAATTCCTCTATAAAGGCTCAGAAAAATTTGCTAAACCCCCCAAAAAATACCTTTACTCTCTCTTTCTTCATTACTCAATCTAA>10100
1124>----->1124
239>TTACAGCTAGAACGAATTCCTCTATAAAGGCTCAGAAAAATTTGCTAAACCCCCCAAAAAATACCTTTACTCTCTCTTTCTTCATTACTCAATCTAA>140

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
10101>TTTCTCTCTTTATCATCTCTTGTCTGTGATGTTCAACGATGCCATAAGAAAAAAGTGAACACCTTAAAGTACTGTATAAATCTTATACAAATTTT>10200
1124>----->1124
139>TTTCTCTCTTTATCATCTCTTGTCTGTGATGTTCAACGATGCCATAAGAAAAAAGTGAACACCTTAAAGTACTGTATAAATCTTATACAAATTTT>40

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
10201>TTTTGGAAGAAAGGCTATATTTTAAAGAAATCAAGCAAAAGTAGATCTCGGAACCCAGCTTTTctgtacaaagtgtgtgatgcttggcgccgtc>10300
1124>----->1124
39>TTTTGGAAGAAAGGCTATATTTTAAAGAAATCAAGCAAAAGTAGATCTCGGAACCCAGCTTTTctgtacaaagtgtgtgatgcttggcgccgtc>1
1124>----->1124

      x      x      x      x      x      x      x      x      x      x      x      x      x      x      x
10301>gagggggggccggtacccggggtacccaaatggtccccaagaagaagaggaaggtgatcccggtgaggaattatctgatcgtgtgagcaag>10400
1124>----->1124

```

Fig. 48. Alignment results between the *pMT589::pERF114* expected sequence and the sequencing result of the DNA fragment amplified with the 1769 and 1770 primer pair.

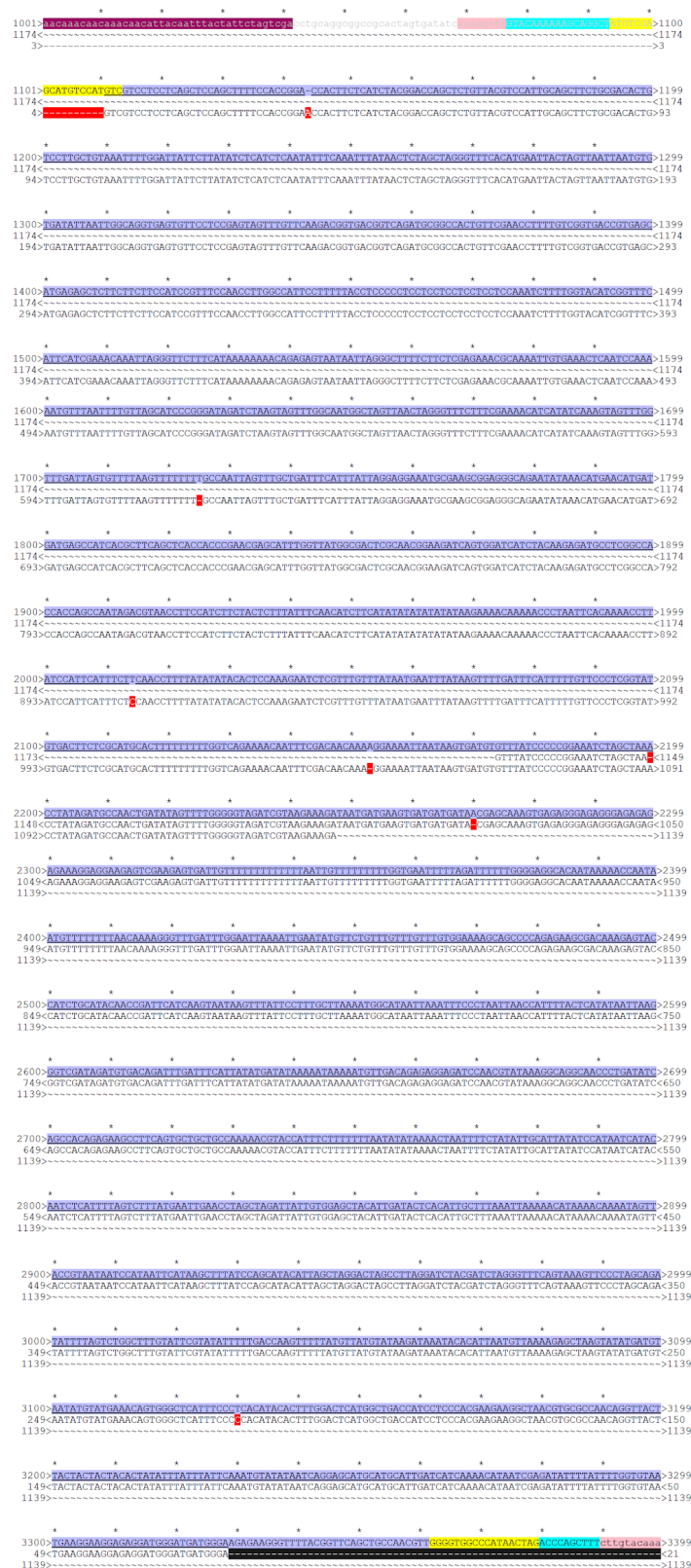


Fig. 49. Alignment results between the *pB2GW7::YAB3* expected sequence and the sequencing result of the DNA fragment amplified with the 1767, 1768 and 1777 primers.



Fig. 50. Alignment results between the *pBGWFS7::pYAB3* expected sequence and the sequencing result of the DNA fragment amplified with the 1771, 1772 and 1779 primers.

```

*      *      *      *      *      *      *      *      *      *
6896>ttaccctatctgtttgtgattatttcttgcagatccgaattccgtaccatgccctcagcaagatcaagtgcacgttcgatctcgacgtccccacgga>6995
9500>ttaccctatctgtttgtgattatttcttgcagatccgaattccgtaccatgccctcagcaagatcaagtgcacgttcgatctcgacgtccccacgga>9599

*      *      *      *      *      *      *      *      *      *
6996>aatcccaactggacggacagcgtttcttgaataataatcttgatcgatgcggccggcaatggcgccgtctctcgaggccgggagacggcgccatcgat>7095
9600>AATCCCACTGGACGGACAGCGTCTTGAATATAATCTTGATCGATGCGGCCGCCGCAATGGCGCGCTCTTCGAGGCCGGGAGACGGCGGCCATCGTG>9699

*      *      *      *      *      *      *      *      *      *
7096>gctgcgctgacgcgcgtcattgcgcgacggcggtgagagcggcgccgctctgcgtgccgcgcccgccgctggtggtgctgccgccggcgccg>7195
9700>GCTGCGCTGACGCGCGTCAATTGCGACGGCGGGCGTGGAGCGCGCGCGCGCTCTGCGTGCCGCCGCCCGCGCGTCTGTTGTTGCTGCCGCCGGCGCG>9799

*      *      *      *      *      *      *      *      *      *
7196>gaacagggcgccggcgccgctcagcgtttgcgcgggagaggaatggtcgccgtcgtctccgtaccctctgatttattgctccatcctatgctgctcag>7295
9800>GAACAGGCGCGGGCGCGCGTTCGACGTTGCGCGGGAGGAGAGATGTCGGCGCTGCTCTCGGTACCTCTGTTTGTGCTCCATCTGTGCTGCTCGAG>9899

*      *      *      *      *      *      *      *      *      *
7296>cccggaacggatgatgatgatgtgtttatgtttgtgtgtactgcgatgtttaatttacaggggagatcacgcggcggggcaaacgtggccgag>7395
9900>CGCGGAAACGGATGATGATGATGTGTGTTATGTGTGTGTACTGCGATGTTAATTACAGCGGAGATCacgcggcggggcaaacgtggccgag>9999

*      *      *      *      *      *      *      *      *      *
R R Y R G V R R R P W G K W A A E I R D
CGCaggTACcgCGGgtgAGGcgCGAccgTGGggcAAGtggGCGcgGAGatcAGGgacC
7396>acgcggcgccggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggt>7495
10000>cgcgcgccgcggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggt>10099

*      *      *      *      *      *      *      *      *      *
P R K A A R V W L G T F R T A E D A A R A Y D A A A L R F R G R R A
CGaggAAGcgCGcgGCTctggCTGggcACcttcCGCaccGCCgagGACgcgGCGcgGCGctacGACgccGCCgcgCTAcgeTTCcgCGGcgCGCgc
7496>cgaggaaagcgccgctctggttgggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggt>7595
10100>cgaggaaagcgccgctctggttgggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggt>10199

*      *      *      *      *      *      *      *      *      *
K L N F P E E V S R P W Q G H D V D H M S C S P P S I S N A R F L
cAAGctcAActtcccGagGAGgtgTCCcgCCGtggCAGggtCACgatGTCgatCACatgTCCtgcTCGcgCCGtgcATCtgcAACgctCGctttCTG
7596>aaagctcaacttccccgagggaggtgtcccgcccgaggaggtacagatgtcagatcacatgtcgtgctgcgcgcgtcgtatctcgaacgctcgtttctg>7695
10200>aaagctcaacttccccgagggaggtgtcccgcccgaggaggtacagatgtcagatcacatgtcgtgctgcgcgcgtcgtatctcgaacgctcgtttctg>10299

*      *      *      *      *      *      *      *      *      *
G S W T F G P P Q P
ggtTCgtggACcttcGGAacgCCGagCCG
7696>gttctgtgagccttcggacggcgccgagccgcatcgccgttcgtcgccggcgccgagcactctgcttgggtgggagccatggaggcaacggagcagaga>7795
10300>GGTTCGTGGACCTTCGACCGCCGACCGCCATCGCGGTCCGTGCGCGCGCGCGCGACGACTCTGCTTGGTGGGAGCCATGGAGGCAACGGAGCAGAGA>10399

*      *      *      *      *      *      *      *      *      *
7796>acgggaggggaatagcgccgagacaaaaagggctggagagatgggaatcgtcgtcccggttactcctagtccacagaacttgagtcgacccatggaagct>7895
10400>ACGGGAGGGAATAGCGCGGAGACAAAAGGGCTGGAGAGATGGGAATCGTCTGTCGGGTACTCTAGTCCACAGAACTTGAGtcgacccatggaagct>10499

*      *      *      *      *      *      *      *      *      *
7896>tctagactagtgaactcgaatttcccgatcggttcaaacatttggcaataaagttttcttaagattgaatcctgttgcgggttcttgcgatgattatcata>7995
10500>tctagactagtgaactcgaatttcccgatcggttcaaacatttggcaataaagttttcttaagattgaatcctgttgcgggttcttgcgatgattatcata>10599

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Fig. 51. Alignment results between the *pBY02::ERF98* expected sequence and the sequencing result of the DNA fragment amplified with the 29 and 30 primer pair.

[illegible]

Fig. 52. Alignment results between the *pBY02::BIP2* expected sequence and the sequencing result of the DNA fragment amplified with the 31 and 32 primer pair.



Fig. 53. Alignment results between the *pAG731::PE YABBY7* expected sequence and the sequencing result of the DNA fragment amplified with the primer 50.

```

101>aataaacttattacTTGATGAATCGGTTGTATGCAGATGGTACTCTTTGTGCCTTCTCTGGGGctgcttttccacaaacaaacaaacagaaacatttcaa>1200
1>~~~~~5TTTGGCTGCT>11

1201>ttttaattccaaatcaaaccttttggtaaaaaaacattattggtttttattgtgcctcccccacaaatctaaaaattccacaaaaaaagaattaaa>1300
12>GGGGCAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAGCTGTGTGCCCGTCTCACTGGTGAAAGAAAAACCACC>111

1301>aaaaaaaaaacaatcactcttgcactcttctccttctctctctcctctcctctcactttgtgcgttatcatcatcaacttcatcattatctttctta>1400
112>CAGTACATTAAAAACGTCGCAATGTGTTATTAAAGTTGTCTAAGCGTCAATTGTGTTACACCACAATATATCCTTCATCACTTCATCATATCTTCTTA>211

1401>cgatctacccccaaaaactatcagttggcatctatagtttagctagatttccgggggataaacacatcacttataattttcccttttgttgcgaaat>1500
212>CGATCTACCCCCAAAACTATATCAGTTGGCATCTATAGGTTTAGCTAGATTTCGGGGGATAAACAATCACTTATTAATTTTCCTTTTGTGTGCGAAAT>311

1501>tgttttctgacccccaaaaaagtgcacgcgagaagtcacataccgaggggaacaaaaatgaaatcaaaacttataaaattcattataaacacacagagattct>1600
312>TGTTTTCTGACCAAAAAAAGTGCATGCGAGAAGTCATACCGAGGGGAACAAAAATGAAATCAAAACTTATAAAATCATTATAAACCAACGAGATTCT>411

1601>ttggagtgatatataaaaaggttgaaagaaatgaatggataaggttttggtaattagggttttgttttcttatatatatatatgaagatgttgaaata>1700
412>TTGGAGTGTATATATAAAAGGTTGAAGAAATGAATGGATAAGGTTTGTGAATTAGGGTTTGTGTTTCTTATATATATATATATGAAGATGTTGAAATA>511

1701>aagagtagaagatggaaggttacgTCTATTGGCTGGTGGTGGCCGAGGCATCTCTTGTAGATATCCACTGATCTCCGTTGCGAGTGCGCATAACCCAA>1800
512>AAGAGTAGAAGATGGAAGGTTACGCTCTATTGGCTGGTGGTGGCCGAGGCATCTC~~~~~>565

1801>TGCTCGTTTCGGGTGGTGAGCTGAAGCGTGAATGGCTCATCATCATGTTCATGTTTATATCTGCCCTCCGCTTCGCATTTCCTCetaataaatgaaatcaq>1900
565>~~~~~>565

```

Fig. 54. Alignment results between the *mt* band for the *yab3* arabidopsis mutant line with the *AtYAB3* sequence.