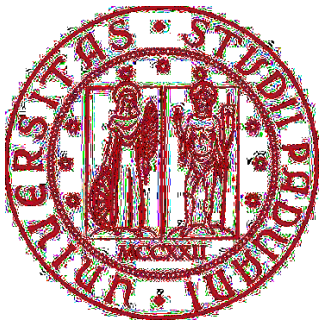

UNIVERSITÀ DEGLI STUDI DI PADOVA
Department of Agronomy, Food, Natural Resources, Animals and Environment

Second Cycle Degree (MSc) in Sustainable Agriculture

"New insights to a MYB candidate gene for the induction of male sterility in *Petunia x hybrida* through a CRISPR-edited DNA-free by transient transformation system."

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ABSTRACT

The inability of a plant to produce pollen, male gametes or functional anthers is known as male sterility (MS). The implementation of the MS trait into parental lines in plant breeding programs is an important task in many horticultural crops, to reduce the cost of hybrid seed production and to ensure varietal purity for the generation of F1 hybrids. In ornamentals, the introgression of MS traits for the production of F1 hybrids has not received much attention because propagation is usually done by agamic methods. However, vegetative propagation allows the direct propagation and commercialization of male-sterile ornamentals, which could be very beneficial for several reasons, such as for example the removing pollen allergens. Recent advances in genome editing (GE) technology, based on CRISPR/Cas-system, have ushered in a new era, also linked to progress in the acquisition of first-generation non-transgenic modified plants and the development of foreign DNA-free editing techniques. Based on these assumptions, we describe the setting of the initial stages for the development of a CRISPR/Cas9-based breeding strategy for the implementation of the MS trait in Petunia x hybrida hort. ex E. Vilm, a model system of the Solanaceae family and always a point of reference in ornamental research. The general objective is to create an efficient protoplast transfection system for obtaining CRISPR/Cas-edited DNA-free plant material, mediating direct delivery of a pre-assembled ribonucleoprotein (RNP) complex consisting of pure Cas9 protein and in vitro synthesized single guide RNA (SgRNA) molecules. In particular, in this master thesis the results and ongoing work on targeting a Petunia MYB candidate gene are described: an expression kinetic analysis of target gene in vegetative tissues and flower buds at different phenological stages has suggested its potential importance in the formation and development of relative anther tissues and pollen. Furthermore, several SgRNAs were then identified and selected based on their potential ability to target the investigated sequence at the CDS level and the predicted TSS region. In addition, several protocols for protoplast isolation and transfection from petunia system were tested and discussed.

LIST OF ABBREVIATIONS

aa- Amino acid

Amp- Ampicillin

BF- Bright field

bHLH- Basic-helix-loop-helix protein

bp- Base pair

CCD- Carotenoid cleavage dioxygenase

CDS- Coding sequence DNA

CMS- Cytoplasmic male sterility

CRISPR- Clustered Regularly Interspaced Short Palindromic Repeats

CYP703A2- Cytochrome P450

°C- degrees Celsius

DAE- Days after emergence

ddH2O- Distilled and deionized water

DNA- Deoxyribonucleic acid

DSB- Double stranded break

EGMS- Environment sensitive genic male sterility

FDA- Fluorescein diacetate

FW- Fresh weight

GE- Genome editing

GFP- Green Fluorescence Protein

GMS- Genic male sterility

HDR- Homology-directed repair

HGMS- Humidity sensitivity genic male sterility

IAA- Indole acetic acid

kb- Kilobase

l- Litre

LB- Luria-Bertani broth

min- Minutes

mg- Milligrams

ml- Milliliter

mM- Millimolar

MS- Male sterile

MS medium- Murashige and Skoog medium

MS2- Male sterile2

mtDNA- Mitochondrial DNA

NHEJ- Non-homologous end joining

NMS- Nuclear male sterility

ng- Nano gram

ORF- Open reading frame

PCR- Polymerase chain reaction

PCD- Programmed cell death

PEG- Polyethylene glycol

PGMS- Photoperiod-sensitive genic male sterile

PPR- Pentatricopeptide repeat

PTGMS- Photo–thermos-sensitive genetic male sterility

RF- Restorer of fertility

RNA- Ribonucleic acid

RNP- Ribonucleoprotein

Rpm- Revolution per minute

RT-PCR- Reverse transcription PCR

sec- Seconds

TALEN- Transcription activator-like effector nucleases

T-DNA- Transfer DNA

TGMS- Temperature-sensitive genic male sterile

TSS- Transcriptional start sites

µg- Microgram

µl- Microliter

µM- Micromolar

WA- Wild Abortive

X Gal- 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside

ZFN- Zinc-finger nucleases

1. INTRODUCTION

The ornamental plants are extremely valuable due to their aesthetic value, therapeutic importance, and beauty. The ornamental industry is characterized by its diversity, primarily deals with non-food horticultural applications, where the final product does not undergo further processing for example wood. (Chandler Stephen, F. 2003). Over the past century, the breeding of crops and ornamental plants has become a growing business. Ever since the explorers in Europe start gathering plants from all over the world, breeders played their role by exploiting variation, which could be developed by mutation breeding and cross breeding, as a result the range and diversity of ornamental plants expanded enormously and now thousands of varieties of ornamental most important cut-flowers, pot plants, hanging plants, bedding plants and shrubs are available (Chandler et al., 2012). According to the estimate of (Johann van Huylenbroeck 2022) there are currently between 85,000 to 99,000 species of ornamental plants in the world including their wild relatives. It shows the enormous potential of natural variation and germplasm, as well as the need to preserve ornamental plant's genetic resources for breeding and future development. The anticipated worldwide production value of ornamental plants comprises about €50 billion, corresponding to an estimated global consumer consumption between €100 to €150 billion. (Henrik et al., 2012). The main areas of both production and consumption of ornamental plants are Europe and the United States with a fast growing ornamental plant industry in Japan and China. Various plants are classified as ornamental and are divided into several categories, such as bedding plants, trees, shrubs, indoor, potted plants, ornamental grasses, lawn or turf grasses, and cut flowering plants. Tulips, lilies, roses, carnations, chrysanthemums, and petunias are some of the decorative plants that are grown for their beauty. These plants are used by geneticists and plant breeders to improve a range of features, including post-harvest longevity, tolerance to biotic and abiotic stresses, flower's color, shape, and fragrance and traits that affect seed production, like male sterility. These improvements of traits in various ornamental plants became possible only with emerging advancements in the field of plant molecular biology, as it provides significant potential for enhancing ornamental plant breeding through the application of recombinant DNA technology. Since, conventional breeding and plant improvement techniques have become inadequate to keep up with progression and quality demands, therefore advanced breeding strategies are increasingly adopted (Chandler S, Tanaka Y 2007). In the past few years genetic engineering has allowed the precise modifications of individual traits, thus enabling the expansion

of gene pool since desirable genes from unrelated species or even organisms can be used for crop improvement. To overcome the narrow genetic variation in current ornamental plant breeding programs, genome-scale studies of large germplasm panels have served to identify important genetic materials, to study genomic variation dynamics during domestication and selective breeding. **Figure 1** represents the graphical data of the sequenced genomes for various ornamental plant from the year 2012 to 2020 (Zheng et al., 2021). However, the availability of data on various sequenced genomes and genome editing approaches, has significantly enhanced the quality of cultivars of ornamental plants through trait modifications (Tanaka et al., 2010). In contrast, more than 30 genera of different transgenic ornamental plants have been generated using various transformation methods to date (Nishihara et al, 2011).

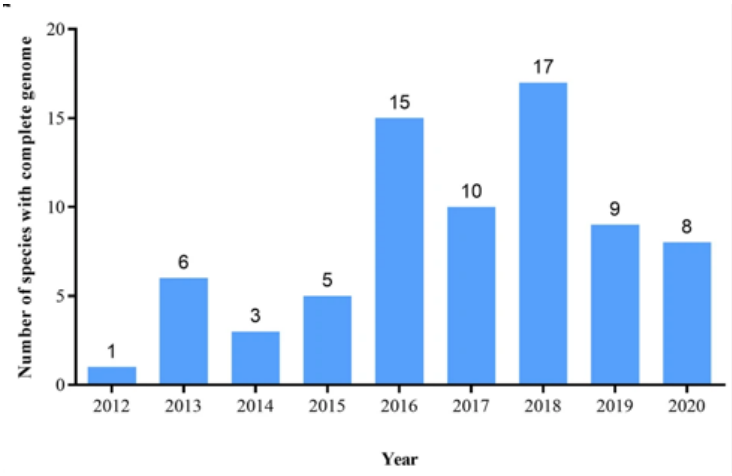


Figure 1. Statistical data on ornamental plant species with sequenced genomes from 2012 to 2020 (Zheng et al., 2021).

1.1 *Petunia*: a model plant of floriculture species

Petunia is a commonly grown ornamental flower known globally one of the most important species in the floriculture trade. It is a member of Solanaceae family commonly known as "Nightshade" family. Petunia is a genus comprising of approximately 35 species. It is native to South America and was brought to Europe in the 19th century when plants with purple flowers were taken there and planted in European gardens. *Petunia hybrida* is an herbaceous plant with generally oval-shaped leaves and smooth margins, and with fine sticky hairs. The flowers are

funnel-shaped, consisting of five fused or partially fused petals and five green sepals. The commercially produced *P. hybida* originated from the hybridization of a white-flowered, moth-pollinated *P. axillaris* with different species within the *P. integrifolia* clade, which includes closely adjacent bee-pollinated species and subspecies. *Petunia hybrida* is a fertile, diploid, annual hybrid species and its varieties have same *2n* chromosome number as the parental species. Since, Petunia species can be crossed perfectly with one another and yield normal diploid progeny thus, varieties of *P. hybrida* do not suffer from related genetic complexities found in hybrids that are allotetraploid (Nishihara et al., 2011; Alisawi et al., 2023).The genome size of *P. hybrida* is 1.4 GB (Bombarely et al., 2016) which is larger than other species in Solanaceae family like potato and tomato (850-900 Mb) (Tomato Genome Consortium, 2012), but smaller than the size of hot pepper (Kim et al., 2014). Among floriculture crops, petunias are unique in that they possess sophisticated molecular genetics, extensive linkage maps, transformation/regeneration methods and a deep comprehension of the taxonomic and cytogenetic relationships among various taxa *P. hybrida* along with its parental taxa is considered as a model specie for studying plant growth and development (Griesbach et al., 2007).

1.2 The male sterility (MS)

In plants, male sterility (MS) refers to the inability to produce or release viable pollen. This occurs due to the unsuccessful formation or development of functional gametes, stamens or microspore, however fertility of female remains unaffected. Male sterile plants can therefore be fertilized by male fertile plants, but they are not capable of self-pollination. The mechanism of plant MS has traditionally been used to produce F1 hybrid seeds. One of the main goals of F1 hybrid production and marketing is the establishment of male sterile lines in plant species that are largely self-fertilizing, it is possible to take advantage of heterosis by blocking selfing (Longin et al., 2012; Kim and Zhang, 2018; Li et al., 2022; Ramlal et al., 2022). Historically, physical emasculation by chemical, mechanical, or manual means was the primary method used to prevent significant proportions of progeny derived from self-pollination, even in species primarily relying on cross-pollination. Therefore, an important benefit of using MS lines is reduction in time, cost and effort involved in these emasculation treatments (Colombo and Galmarini, 2017). Due to these factors, MS lines have been established in many economically valuable species, (Abbas et al., 2021; Wang et al., 2023) particularly in horticultural crops and ornamental plants (Yamagishi and Bhat, 2014; Barcaccia et al., 2016; Khan and Isshiki, 2016; Jindal et al., 2019; Singh and Khar, 2021). Male

sterility (MS) is primarily caused by Mendelian inheritance, which is controlled by either nuclear gene alone or by the coordinated interaction of cytoplasmic and nuclear genes (Kaul, 1988). The three-line breeding system or cytoplasmic male sterility (CMS) is dependent on loci found in the mitochondrial genome. MS is maternally inherited therefore all progeny deriving from plants carrying S locus (sterile) cytoplasmic inherit male sterility (Chen et al., 2017). CMS condition can be overcome by nuclear genes that are functional in dominant conditions (Jindal et al., 2019), defined as restorers of fertility (Rf) and can suppress or downregulate the CMS genes and revert male sterility (Kim et al., 2018). Conversely, genic male sterility (GMS), also referred to as nuclear male sterility (NMS), or two-lines breeding system, is typically controlled by single nuclear genes, predominantly through recessive alleles (ms) (Singh et al., 2019).

1.2.1 The male sterility in crop species

MS was first observed by the German botanist Joseph Gottlieb Kolreuter in 1763. (Mayr E, 1986) and it has been reported in more than 610 plant species (Kaul MLH, 1988). Male sterility has been utilized in crop hybrid breeding for an extended period. Enhancing crop yield, adaptability, and resilience to biotic and abiotic stresses through heterosis has been used as a traditional method in crop improvement (Hickey, 2019; Tester, 2010). Utilization of heterosis has revolutionized plant breeding during the past few decades, leading to yield gains of 3.5–200% in various crop species (Kim, 2018) and helped in encountering the challenges of global food security. (Saxena et al., 2015). MS existing either as a spontaneous machinery (Hanson and Bentolila 2004) or it can be created through experimental means like induced mutations, wide/ inter-specific hybridization, protoplasmic fusion and genetic engineering (Yamagishi and Bhat 2014; Wang et al. 2013; Singh et al. 2015).

CMS in crop species

In several crop species, including rice, sorghum, sugar beet, sunflower, radish, cabbage, carrot, maize, onion, and tobacco, plant breeders have successfully searched such systems and large quantities of commercial F1 hybrid seeds are currently being produced using CMS system all over the world.

Maize a member of the grass family (Poaceae), represents one of the earliest crops to utilize CGMS systems for hybrid production. Three types of CMS systems have been identified in this crop,

namely CMS-T (Texas), CMS-C (Charrua), and CMS-S (USDA) (Gautam et al., 2023). The **CMS-T** type was the first to be successfully used in large-scale seed production, as its molecular basis has been extensively studied (Yang et al., 2022). In CMS-T, a chimeric gene, *T-urf13*, is responsible for male sterility. In this system, pollen abortion occurs during microspore development in meiosis, resulting in relatively stable sterility (Dewey et al., 1986, 1987). However dominant nuclear genes *Rf1* and *Rf2* located on chromosome 3 and 9 respectively can restore full fertility in CMS-T type of maize plants (Wise et al., 1999). **CMS-C** is the second type of MS identified in maize which is widely used for seed production (Dewey et al. 1991). Male sterility in CMS-C maize is caused by the mitochondrial gene *atp6c* which results in premature tapetal cell death and pollen abortion (Yang et al. 2022a, b). *Rf4* and *Rf5*, are the two restorer genes, which collectively contribute to fertility restoration in the CMS-C sterile line through their additive impact. These genes are present on chromosomes 8 and 5 respectively. (Jaqueth et al. 2020; Kheyr-Pour et al. 1981; Sisco 1991). *Rf4* is a dominant restorer gene which has the ability to restore all CMS-C lines, whereas, *Rf5* can only restore CMS-C in which *Rf-I* gene is deleted. *Rf-I* gene can act as a suppressor inhibiting the fertility restoration function of *Rf5*. (Jaqueth et al. 2020). The third type of MS found in maize crop is **CMS-S**. In the recent study it has been reported that the male sterility of CMS-S maize is associated with stage-specific *orf355* gene expression, mitochondrial abnormalities at developmental stage 10, and nuclear-encoded transcription factor *ZmDREB1.7* that is highly expressed in sterile microspores at the large vacuole stage promotes the expression of CMS gene *orf355* in maize plant containing S type of cytoplasm (Xiao et al., 2020). However, the major restorer gene identified in CMS-S sterile lines is *Rf3*, located on the long arm of chromosome 2 (Kamps & Chase, 1997; Laughnan & Gabay, 1978; Zhang, Wang & Zheng, 2006; Xu et al., 2009). *Rf3* restores fertility through post-transcriptional modification which is accomplished by cleaving the 1.6-kb transcripts associated with *orf355* and ultimately restores fertility (Gallagher et al. 2002; Qin et al. 2021; Xiao et al. 2022b).

Rice is a primary staple food crop, hybrid rice, produced either by three-line or two-line strategy for hybrid seed production, yields approximately 10%–20% more grains compared to conventional rice breeding (Cheng et al., 2007) 74. However, rice cytoplasmic male sterility (CMS) provides a useful model for studying genetic interactions within plant nuclei due to its hereditary characteristic of having a non-functional male gametophyte and number of genes involved in the control of CMS have been used and reported successfully (Chen and Liu, 2014). In the recent

study, it has been reported that a gene named *OsRab7* regulates vesicle trafficking in rice and the functional loss of *OsRab7* significantly reduced pollen fertility and setting rate in comparison to the wild type. However, it was also reported that the *rab7* mutant exhibited premature tapetum and abnormal microspores development. The expression of critical genes involved in tapetum development (*OsMYB103*, *OsPTC1*, *OsEAT1* and *OsAP25*) (Lei et al., 2022; Li et al., 2011; Niu et al., 2013) and pollen development (*OsMSP1*, *OsDTM1* and *OsC4)* (Li et al., 2006; Nonomura et al., 2003; Yi et al., 2012) significantly decreased in the anthers of *rab7* mutant compared to the wild type therefore resulting in CMS in rice (Ying et al., 2024). More than 60 types of CMS systems based on the origin of the cytoplasm have been developed in rice (Li et al., 2007) 76. Important CMS lines in rice are CMS-BT (Chinsurah Boro II), and Wild Abortive (WA). CMS-WA has been used extensively to breed hybrid rice cultivars (Kim and Zhang 2018; Toriyama 2021). Genes responsible for CMS-WA is *WA352,* it interacts with COX11 to repress its function, inducing premature programmed cell death (PCD) in tapetal cells and causing pollen abortion (Luo et al., 2013). The fertility in CMS-WA can be restored by *Rf3* and *Rf4* genes in a sporophytic way (Tang et al. 2014; Zhang et al. 1997, 2002). In CMS-BT line gene responsible for male sterility is *orf79* which results in mitochondrial dysfunction and causes severe pollen abortion (Kim and Zhang 2018; Kazama et al., 2016). However, nuclear genes *Rf1a* and *Rf1b* encode for Pentatricopeptide repeat (PPR) proteins and this protein is responsible for restoring fertility of CMS-BT rice line.

GMS in crop species

GMS is exclusively caused by nuclear genes. It is commonly observed in angiosperms and is reported in nearly every major crop species. GMS can arise from mutations however, genes coding transcription factors are capable of modifying the expression of genes involved in reproductive processes such as pollen/stamen development. resulting in a wide range of GMS phenotypes (Jeong et al., 2014; Sawhney et al., 1997). Many GMS mutants are not suitable for hybrid seed production because their male-sterility traits cannot be maintained efficiently. MS can be affected by environmental factors (also known as EGMS). However, the discovery of EGMS mutants has enabled some GMS traits to be used for hybrid crop breeding. For cereal crops such as wheat and rice temperature-sensitive genic male sterile (TGMS) and photoperiod-sensitive genic male sterile

(PGMS) lines have been created. The pollen fertility of EGMS lines changes in response to day length and temperature (Virmani et al., 2001).

Wheat (*Triticum aestivum L.*) is one of the most extensively cultivated and serves as a crucial cereal crop globally (Ray et al., 2013). Wheat is a self-pollinated crop with small flower organs and removing stamens is a challenging task. Therefore, using MS lines as the female parent can enhance seed production efficiency, ensure seed purity. TGMS and PTGMS variants of wheat have been identified in China. Four mutants that can be classified as TGMS have been reported. YanZhan 4110S is a line where male fertility is temperature-sensitive during the late uni-nucleate stage of pollen development, becoming completely male sterile at temperatures above 20 °C. Two genes, *TaMUT11* and *TaSF3*, have been linked to TGMS in YanZhan 4110S (Yang et al., 2021). Another TGMS line that has been shown to be a spontaneous mutation of the fertile wheat line BNY-F is BNY-S. During the spikelet differentiation stage, BNY-S displays male sterility at temperatures below 10 °C, while at temperatures over 10 °C, male fertility develops. However, a single recessive gene *wtms1* controls sterility in BNY-S (Xing et al., 2003). BS20T is also a TGMS line used for the breeding of hybrid wheat in northern China [41]. t*msBS20T* is a single gene located on chromosome 2BL, which is responsible for MS in BS20T (Ru et al., 2015). Another TGMS line identified in wheat is BNS, it exhibits male MS in the temperature range of 7.4 °C to 11.4 °C but is male fertile at temperature higher than 11.4 °C. According to research the main cause of sterility in BNS might be the early degradation of tapetum hindering the normal development of microspores, thereby leading to pollen sterility (Li et al., 2009; Niu et al., 2019).

In addition to TGMS mutants, several PTGMS lines have been identified in wheat. For example, BS366 exhibits male sterility when subjected to a condition of 10 °C with 12–14 h daytime during pollen development (Yuan et al., 2020). Other photo–thermos-sensitive genetic male sterility (PTGMS) lines reported in wheat are C49S (Zhang et al., 2003), K78S, C412S (Mujun et al., 2006), BS210 (Sun et al., 2017) and XN291S (Dong et al., 2012; Singh et al., 2021).

Rice, but in particular EGMS lines account for 20% of the hybrid rice cultivation area (Li et al., 2007). The basis of two-line hybrid breeding consists of the EGMS male sterile genes. Based on the main factors affecting fertility in rice EGMS can be divided into PGMS, TGMS and Humidity sensitivity genic male sterility (HGMS). TGMS lines constitute a very important source for hybrid rice breeding. The *TMS5* is a key sterility-fertility transition gene, that has been extensively studied

for its role in fertility under varying temperature conditions. *TMS5* encodes RNase ZS1, which regulates the accumulation of ubiquitin-60S ribosomal protein L40 (UbL40) mRNA. RNase ZS1 cleaves UbL40 mRNAs to maintain fertility. In *TMS5* mutants, the absence of RNase ZS1 leads to an over accumulation of unprocessed UbL40 mRNAs at high temperatures, resulting in defective pollen and male sterility (Zhou et al., 2014). In PGMS lines the photoperiod is considered as the key regulator. Lines created via the PGMS-system are male sterile under long-day (LD) conditions and restore male fertility under short-day (SD) conditions. The main PGMS genes identified in rice are *pms1, pms2, pms3* and *pms4*, which are mainly derived from japonica rice cultivar NK58S. Under long-day conditions, pollen abortion in NK58S starts at the early pollen mother cell (PMC) stage and persists throughout the pollen development. This is associated with abnormal tapetum formation (Shi et al. 2009), which causes a gradual degeneration process that impedes the availability of nutrients to developing microspores. PCD also plays a role in this premature tapetum degeneration, occurring earlier in NK58S resulting in male sterility (Ding et al. 2012a). HGMS is male sterile in low humidity environment and fertile in high humidity environment. Recent research has identified HGMS lines in rice, and related genes such as *OsCER1* (*OsGL1-4*), *OsOSC12*, and *OsHMS1* have been reported. *OsCER1* is specifically expressed in the tapetum of rice anther development stage 10, 11. It plays a key role in ultra-long chain lipid biosynthesis in rice, affecting plastid development and PCD in rice tapetum hence causing male sterility (Ariizumi et al., 2003).

1.2.2 The male sterility in ornamental species

Breeders of ornamental plants are always looking for cutting-edge technology that could help them cut expenses, enhance the quality of their products, and increase their selection (Eeckhaut et al., 2006). The breeding process for many ornamentals has been expedited using in situ and in vitro haploid/double haploid techniques. Significant progress has been achieved through ovule culture, embryo-rescue methods, and backcrossing (Datta et al., 2022). However, in ornamental plant research the phenomenon of male sterility which is commonly used for producing F1 hybrid, an ultimate goal of the breeder, has not been deeply investigated to date. Furthermore, there are very few examples of some ornamentals like sunflower, chrysanthemum, petunia and cotton in which MS has been studied (Budar et al., 2001).

CMS in petunia was the result of a purposeful breeding project in which interspecific hybrids were created based on the successful production of male sterility in tobacco (Clayton 1950). CMS trait

was successfully transferred into a wide variety of petunia specie by repeated backcrosses. In case of *P. hybrida* the gene responsible for CMS is *S-pcf*, it is composed of 35 codons of the open reading frame of *atp9*, portions of two of the exons of *cox2* (comprising 156 codons), and 207 codons of unknown origin (Young and Hanson 1987). Sporogenous and tapetal cells in anthers of CMS lines display abnormalities during meiosis, ultimately resulting in abortion of pollen. Petunia lines carrying the CMS cytoplasm can be restored to normal male fertility by the presence of a single copy of a dominant nuclear *Rf* gene, which in Petunia appears to involve interactions between the RF protein and *pcf* transcript (Gillman et al., 2009).

In sunflower only one CMS system, the so called PET1-cytoplasm, resulting from the interspecific hybridization of *Helianthus petiolaris* with *Helianthus annuus* has so far been used for commercial hybrid breeding (Sajer et al., 2020), although more than 70 CMS sources have been reported so far. CMS in sunflower is associated with the insertion into the mitochondrial DNA of a novel *OrfH522* located 3' to the *atpA* gene. In a research study the mitochondrial DNA (mtDNA) organization of fertile (HA89) and male sterile CMS89 and of *H. annuus* and of *H. petiolaris* were compared and within the entire rearranged mtDNA region only the *atpA* locus shows differences in transcript pattern between HA89 and CMS89. The mtDNAs of fertile and male-sterile lines differ by an 11 kb inversion and a 5 kb insertion. However chimeric gene is responsible for the formation of 15 kDa novel polypeptide which is probably responsible for CMS phenotype (Hans Kohler et al., 1991; Moneger et al., 1994).

In contrast to CMS, nuclear male-sterile plants are useful for understanding pollen development, but their use in breeding programs is limited because homozygous male-sterile populations cannot be generated (Williams 1995). To overcome the limitations of nuclear male sterility, in particular the issue MS trait segregation, various strategies have been developed to utilize and regulate nuclear genes for creating and maintaining pollen-sterile plant population. Furthermore, with advancements in genetic engineering, numerous studies now focus on using precision breeding techniques to enhance various traits in ornamental plants, including male sterility.

1.3 New breeding techniques

Conventional breeding is currently the most widely used approach in crop improvement. It has empowered breeders to develop enhanced varieties of numerous crops and has led to increased food security. These improvements have resulted in crops with higher yields, improved nutritional profiles, and increased resistance to biotic and abiotic stresses. However, these conventional breeding methods are labor intensive and it requires a long period to progress from the early stages of screening phenotypes and genotypes to the first crosses into commercial varieties. Breeders are still facing increasing challenges that need to be overcome mainly due to climate change and greater consumer demands. (Miladinovic et al., 2021; Zhang et al., 2019). Recent advancements in sequencing technologies have made it easier to obtain genetic data for a growing number of plant species. These developments, when combined with the progress made in genome editing tools, allow for precise gene manipulation, creating new opportunities to improve crop quality. Genome editing (GE) is defined as a collection of advanced molecular biology techniques that facilitate precise, efficient, and targeted modifications at genomic loci. It provides novel means for engineering crops with enhanced characteristics and improved traits. For two decades use of zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) for genome editing was very common (Christian et al., 2010). These methods use standard sequence-specific nucleases (SSNs), that can be activated to identify specific DNA sequences and produce double-strand breaks (DSBs). These DSBs can be fixed by plants endogenous repair system either by non-homologous end joining (NHEJ) or homology-directed repair (HDR) which can lead to the insertion or deletion of nucleotides, exchanges at the target sites thereby causing gene knockouts (Symington et al., 2011). Genome-editing technologies have been used to produce many gene knockout mutants and some gene replacement and insertion mutants in a wide variety of plants, and many of these mutants have been shown to be useful for crop improvement.

1.3.1 The CRISPR/Cas system as tool for genome editing

The clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9) is derived from the adaptive immunity system of bacterial type II. It is another DSB- based breakthrough technology which was developed and widely used for genome editing in many organisms including plants (Zhu et al., 2016). CRISPR/Cas 9 system is characterized by its simplicity, efficiency, low cost and by its ability to target multiple genes when compared with

other programmable nucleases such as ZFNs and TALENs (Cong et al., 2013; Mali et al., 2013). The CRISPR/Cas9 system has been rapidly exploited in many plant species because of these distinctive traits, and it may provide an effective solution to many problems in plant breeding (Li JF et al., 2013; Nekrasov et al., 2013; Shan et al., 2013). Many crops such as rice maize, wheat, soybean, barley, sorghum, potato, tomato, flax rapeseed, camelina, cotton, cucumber, lettuce, grapes, grapefruit, apple, oranges and watermelon have been edited using this genome editing tool (Zhang et al., 2019).

The main components of CRISPR/Cas9 system are; Cas9 protein, easily engineered single guide RNA complex which recognizes a particular 20 base pair (bp) region in the target genome containing a protospacer-adjacent motif (PAM) sequence, which is required for effective target recognition and precisely create DSBs at target gene loci (Khaoula et al., 2015; S. Kim et al., 2014). These DSBs are repaired either by NHEJ to produce mutations in the targeted site or by HDR if a homologous donor DNA template is present to produce gene fragment (knock-in) or replacement for precise gene editing (Arora et al., 2017). Despite the well-established understanding of CRISPR-Cas9 genome editing system, differences in species have been observed to impact different outputs in terms of accuracy and efficiency. However, in plants different off-target activity and mutations can be produced but it mainly depends on following factors such as cell type, sgRNA design, selection of target site, endonuclease properties and type of DSBs (Bortesi & Fischer, 2015).

1.3.2 The CRISPR/Cas system for male sterility induction

The CRISPR/Cas technology is constantly being improved to increase the accuracy and efficiency of targeting different genes (Arora et al., 2017). Apart from characteristics related to quality, yield and stress response, CRISPR/Cas-based technology offers a novel strategic approach which can be used for exploiting additional crop features related to sterility and fertility. An increasing number of genes involved in the development of male reproductive organs also having putative role in male sterility have been characterized thanks to the growing amount of transcriptomic and proteomic research conducted in recent decades, primarily focused on crop species. However, CRISPR/Cas tool has proved to be an alternative, rapid method in generating MS line through target gene editing both in food crops as well as in ornamental species (Farinati et al., 2023). Among different CRISPR/Cas systems, the most famous Cas9 has been effectively utilized in

developing male sterile line in many significant food crops globally. Investigations carried out on major crops such as maize, rice, wheat, soybean and tomato have indicated that employing collective CRISPR/Cas9 techniques can be useful in generating a pool of mutants showing male sterility characteristics (Liu et al., 2020; Farinati et al., 2023; Yang et al., 2021; Jacobs at al., 2017). Use of CRISPR/Cas9 tool for producing male sterile phenotype proves to be effective particularly by employing a knock-out method to target GMS genes of nuclear origin. This approach is preferred over CMS for producing hybrid seeds since it is easier to use (Qi et al., 2020). CRISPR/Cas9 system accelerate crop breeding cycles as well as precisely improve agronomic traits (Chen et al., 2019). For example, (Li et al., 2016) used CRISPR/Cas9 to target the endogenous CSA gene in rice to produce a photosensitive male-sterile line, providing a straightforward method for producing csa-based rP(T)GMS lines in the two-line hybrid rice system. In tomato (Du et al., 2020) used CRISPR/Cas9 to modify a stamen-specific gene, *SlSTR1*, and created a new tomato male sterile line that could be used for hybrid breeding. Thus CRISPR/Cas system is a convenient method for producing male-sterile lines.

1.3.3 CRISPR/Cas applications in modifying MYB genes for male sterility

MYB proteins are a major family of transcription factors in plants. More than 100 R2R3 MYB members are present in the genome of monocots and dicots (Li et al., 2019). They are crucial for various biological processes, including development, secondary metabolism, signal transduction, disease resistance, and stress response (Dare et al., 2008). Plant MYB family is large, with more than 198 members in Arabidopsis, 234 in grape and 183 in rice (Chen et al., 2006). Structurally, a complete MYB comprises three-parts: the DNA structure binding region (DBD), the transcriptional activation domain, and the negative regulatory region (Ogata et al., 1996). MYB subgroups in plants are classified on the basis of short, conserved sequence motifs. These conserved motifs help in understanding phylogenetic relationships and infer biological functions as they provide essential molecular functions to the proteins that contain them. (Millard et al., 2019; Stracke et al., 2001).

Majority MYB genes are reported as positive regulators of transcription, and for example, specific MYB members are involved in anther development (Zhang et al., 2007) such as *MYB26*, *MYB33*, *MYB32*, *MYB35* and *MYB80.* Among them, *MYB35* and *MYB80* play vital roles in tapetal and pollen development. In Arabidopsis the *MYB80* gene encodes an R2R3 transcription factor

essential for various stages of anther development (Phan et al., 2012). In many crops such as, in canola, wheat, rice, and cotton MYB80 homologs have been identified due to amino acid sequence similarity. While the R2R3 DNA-binding domains of these proteins are highly conserved, whereas the C-terminal region shows greater variability. However, mutations in the first exon of *MYB80* also known as *MYB103* is responsible for male sterility in Arabidopsis. This mutation disrupts tapetum development and callose dissolution in *MYB103*-defective plants (Zhang et al., 2007). Using different approaches such as transfer DNA(T-DNA) knockout, RNA antisense or point mutation, the functional disruption of *MYB80* resulted in partial (in case of antisense lines) or complete male sterility (Higginson et al., 2003; Li et al., 2007; Zhang et al., 2007). Analysis of the gene expression profiles between wild-type flower buds and *AtMYB103* mutant (ms188) revealed that the loss of function of *AtMYB103* blocks several key metabolic and signaling pathways. This finding suggests that *AtMYB103* is a crucial regulator of anther development in Arabidopsis (Zhu et al., 2010). Also in rice for example, the function of MYB proteins in anther development was well studied. According to a recent study, *OsMYB80* was specifically expressed in rice anthers during the tetrad formation and microspore release from the tetrad, this expression was similar to the timing of *AtMYB80* expression during the Arabidopsis anther development (Pan et al., 2020)

1.3.4 CRISPR-edited DNA-free by transient transformation system

As discussed earlier target-specific genome editing facilitated by CRISPR/Cas9 is recognized as a highly effective tool for both fundamental and applied plant research because of its ability to induce mutations at a high frequency while being relatively easy to use (Zhang et al., 2013; Lowder et al., 2015; Ma et al., 2015; Wang et al., 2016). Multiple techniques have been used to develop plants which are CRISPR/Cas9 edited but free from CRISPR constructs and other transgenic elements. However, there are still major challenges faced in terms of commercial applications of CRISPR/Cas technology in agriculture (He and Zhao, 2020). The absence of transgenes in gene-edited plant is essential for commercialization of any CRISPR-edited plants with stable and desirable traits. There are several reasons, transgenes are not allowed in commercially viable gene-edited crop plants. Firstly, cultivating plants containing a CRISPR gene-editing constructs may be potentially hazardous to the environment. Release of pollen or seeds into the environment containing components of CRISPR is not acceptable to the public therefore, gaining approval from government regulatory bodies would be a difficult task (Callaway 2018;

Huang et al. 2016). Secondly, it becomes challenging to evaluate the stability and penetrance of crop traits in the presence of CRISPR constructs because the guide RNA and CRISPR enzyme may continue to edit the existing targets or even off-targets, creating uncertainty in trait expression (Gao and Zhao 2014).

However, the important strategies useful to avoid the maintenance of transgene integration have been discussed in detail by (He and Zhao, 2020). Following CRISPR-mediated mutagenesis, the Cas9 gene and related DNA sequences are typically removed through genetic segregation, which eases public concerns about genetically modified organisms. The major advantage of this method is that it allows the selection of plants without any T-DNA sequence, thus producing plants without foreign DNA even if they were created using transgenic technology. Traditionally, plant transient transformation technology has been widely used as an alternative approach to facilitate rapid and efficient gene function analysis (Chen et al., 2006; Sheen, 2001). Agrobacterium sp. mediated transient transformation (Cui et al., 2017), polyethylene glycol (PEG)-mediated protoplast transfection (Cankar et al., 2022), and particle bombardment (Romano et al., 2003) are a few examples of transient transformation approaches that have shown encouraging results in plant research. Among these techniques, protoplast transient expression system has played a very vital role in proteomics and genomics research, providing a quick and easy way to assess new technologies such as genetic engineering approaches. Transient expression techniques for protoplasts have been developed for various crop species, including monocots, dicots, herbaceous, and woody plants. Some examples are rice (Yang et al., 2014a), barley (Bai et al., 2014), maize (Cao et al., 2014), apple (Maddumage et al., 2002), and grapevine (Zhao et al., 2016). These results highlight the possibility and feasibility of using protoplasts for CRISPR-mediated gene editing, especially in species that have asexual reproduction, heterozygosity, or an extended juvenile phase. Similarly, this strategy also represents the most practical way to directly utilize DNA-free genome editing technologies mediated by CRISPR to improve traits and increase market value, as already experimentally confirmed for food and non-food crops, such as strawberry (Martin-Pizarro et al., 2019; Wilson et al., 2019), potato (Gonzalez et al., 2019; Nicolia et al., 2021; Zhao et al., 2021), lettuce (Woo et al., 2015), chicory (De Bruyn et al., 2020; Cankar et al., 2022), *Nicotiana tabacum* (Lin et al., 2018; Hsu et al., 2019) and ornamental species, such as petunia (Yu et al., 2021b). Protoplast transient expression systems offer a promising and effective approach for

producing CRISPR-edited DNA-free plant material and also for generating male sterile mutants. Since protoplasts are individual cells that have been edited before the first cell division therefore, new plants grown from a single modified protoplast guarantee genetic homogeneity in every cell and the inheritance of modified alleles in the next generation (Farinati et al., 2023).

1.3.5 Potential application of the CRISPR/Cas system in MS ornamental species

The introduction of robust genome editing technology such as CRISPR/Cas holds significant potential, and it is continuously resulting in the progress of floriculture industry by enabling precise genetic modifications (Partap et al., 2023). In ornamental plant research MS has not been deeply studied yet. Applications of CRISPR/Cas system are continuously being tested for the improvement of several traits in ornamentals such as flowers longevity, modified time of flowering and novelty in color range and fragrances. Although conventional breeding approaches such as, polyploidization, mutagenesis, double haploid induction and intra and interspecific hybridization have been extensively utilized to develop new varieties in ornamental species, but drawbacks and limitations are still evident (Regalado et al., 2017; Maluszynski et al., 1995; Kato and Mii, 2012) 181. Many floricultural crops such as carnations, chrysanthemums and roses have high level of heterozygosity, others have long life-cycle (e.g, anthurium), high chromosome numbers (e.g, hibiscus) and large genome size in specie like lilium and chrysanthemums also the availability of limited gene pool for new traits, significantly restrict the application of classical breeding strategies whereas, genome editing approaches are particularly desirable (Anderson, 2006; Bisognin, 2011; Giovannini et al., 2021). However, obtaining transgene-free first-generation modified plants facilitating the development of foreign DNA-free editing techniques would be extremely beneficial in these cases. The practicality of utilizing these techniques in the breeding of ornamental species depends on a number of variables, including the availability of efficient protocols for transformation and regeneration, the structure of plant genomes, and functions of genes. In recent years, advancements in genome sequencing technology played an important role, allowing site-specific mutagenesis on several key genes controlling desirable traits. These findings suggest that applications of CRISPR/Cas9-induced mutagenesis is effective also in ornamental sector (Zhang et al., 2016a; Kishi-Kaboshi et al., 2017; Yan et al., 2019; Yu et al., 2021b) and CRISPR/Cas technology, has been successfully employed to create gene knockouts and induce genetic alterations in ornamentals like *Petunia inflate* and *Petunia hybrida* (Subburaj et al., 2016; Zhang

et al., 2016a; Sun and Kao, 2018; Yu et al., 2021b; Xu et al., 2022), *Chrysanthemum morifolium* (Kishi-Kaboshi et al., 2017), *Dendrobium officinale* (Kui et al., 2017), *Ipomoea nil* (Watanabe et al., 2017), *Lilium longiflorum* and *Lilium pumilum* (Yan et al., 2019), and *Phalaenopsis equestris* (Tong et al., 2020). MS induction and development of male-sterile ornamentals plants holds significant interest for various purposes such as facilitating hybrid seed production, eliminating pollen allergens (i.e., gene escape), minimize the need for deadheading to prolong the flowering period, reallocating resources from seed production to vegetative growth, and enhancing flower longevity and shelf life (Garcia-Sogo et al., 2010). **Figure 2** schematically represents the CRISPR/Cas9 genome editing applications for the ornamental plant improvement.

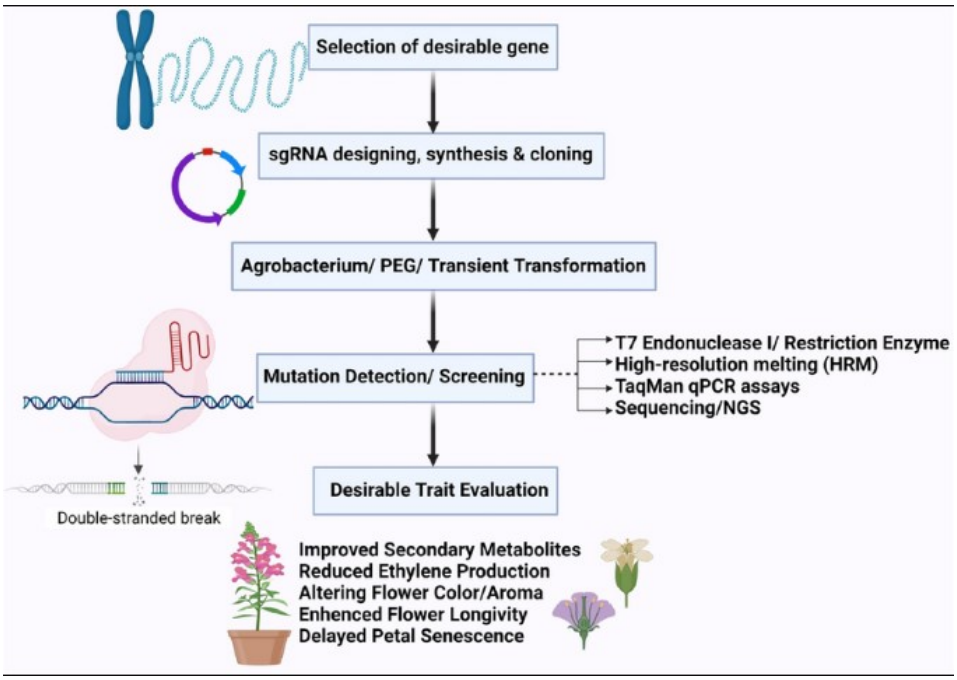


Figure 2. Flowchart depicting steps involved in the improvement of ornamentals using CRISPR/Cas9 technology (Sirohi et al., 2022).

Many studies have been reported that highlights the effectiveness of CRISPR/Cas9 as genome editing tool in major crops and ornamental species. The modification of Carotenoid cleavage dioxygenase (CCD) gene in Ipomoea plant resulted in a 20-fold increase in carotenoid content in the petals of CRISPR edited plants (Watanabe et al., 2018). In *Petunia hybrida* cv. Mirage Rose CRISPR/Cas9 technology was used to modify the *PhACO1* gene. When compared to wild-type lines, mutant lines showed longer flowering times (Xu et al., 2019), and the mutation frequency

was overall 31.5 %. The goal of creating male sterile (MS) lines in ornamental plants and the encouraging results obtained in some cases highlight the potential benefits of incorporating CRISPR/Cas-based technologies for the purpose of genetic improvement in floricultural research.

2. AIM OF THESIS

As described in introduction section, MYB gene family are important regulators of gene transcription in plants. In particular, some members belonging to this gene family have been well characterized since involved in anther and pollen development. For example, in Arabidopsis and rice *MYB80* takes a key role as direct controller of pollen development, and could be a potential target for developing of MS line. Furthermore, the genome editing approach based on CRISPR/Cas9 system has represented a significant molecular tool become very important in precision breeding since it allows precise genetic modifications in target genes involved in specific biological processes. Additionally, protoplast transient expression system has played a relevant role in plant research, resulting in a potential, rapid, and convenient technique for testing new technologies, such as GE approaches.

Based on these presumptions, in this thesis the setting of initial stages for developing a CRISPR/Cas9-based breeding strategy for implementing the MS trait in *Petunia x hybrida* hort. ex E. Vilm, a model system for the Solanaceae family, and always a point of reference in ornamental research, is described. The general objective is to create a temporary protoplast transfection system that is mediated by CRISPR/Cas-edited DNA-free plant material. This will be accomplished by directly delivering a ribonucleoprotein (RNP) preassembled complex that consists of pure Cas9 protein and in vitro synthesized single guide RNA molecules (SgRNA). In particular, I report the results obtained in the targeting of a Petunia MYB candidate gene, the putative orthologue to *AtMYB80*, as well as *OsMYB80*, whose central role in the development of pollen and tapetum was well described in literature. The amplification and isolation of genomic Coding DNA sequence (CDS) and basal promoter regions of target loci were performed confirming the identity degree between amplified regions and reference sequences in databases. Several SgRNAs were then identified and selected by web application, based on their potential ability to target the investigated sequence at CDS level and predicted Transcriptional start sites (TSS) region. The following in vitro SgRNA synthesis/transcription was used to measure the target specificity in order to assess their potential for application in vivo later on. Additionally, a target gene expression kinetics analysis was carried out on vegetative tissues and flower buds at various phenological stages, confirming the gene's expression in stamens at particular stages and indicating its potential significance for the formation and development of relative anther tissues and pollen.

3. MATERIAL AND METHODS

3.1 Plant material and growth conditions

Plants of a *Petunia x hybrida* hort. ex E. Vilm. Clonal line were grown in pots in greenhouse conditions at the experimental farm of University of Padova, Italy (GPS coordinates: 45° 20′ 48.9″ 110 N 11° 57′ 00.3″ E). Internode segments were surface sterilized using 50% bleach and a few drops of Tween-20 for 5 min followed by at least three washes with sterile water, then in vitro transferred in Murashige and Skoog MS medium supplemented with 3.0% sucrose and 0.8% agar. The boxes were placed in plant growth chamber under controlled temperature and light conditions (23 °C, 16 h/8 h photoperiod light/dark).

3.2 Bioinformatics analysis

3.2.1 Sequence analysis

The PaMYB106 (Peaxi162Scf03974g00003.1) amino acidic sequence was downloaded from the *P. axillaris* (Lam.) genome (Bombarely et al., 2016) Solanaceae Genomics Network data base (SGN http://solgenomics.net). The predicted amino acid sequence was aligned to the sequence of previously studied orthologous genes of *Arabidopsis thaliana L.* (Zhang et al., 2007), *Brassica napus L.* (Xu et al., 2014), *Gossypium hirsutum* (Xu et al., 2014). *Oryza sativa L.* (Pan et al., 2020), *Cichorium intybus L.* (Palumbo et al., 2019) and *Lilium Oriental Hybrid* (Sui et al., 2015), available in the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov) database. Using Geneious software (https://www.geneious.com) and the proprietary algorithm, while a Jukes-Cantor Neighbor-Joining tree was generated. A putative basal promoter region of *PaMYB106* was analyzed for the presence of putative TATA box and the prediction of TSS using the Softberry-TSSP software (http://www.softberry.com).

3.2.2 Primers design

Primers for the amplification of CDS and promoter region of PaMYB106 and for the expression study were designed using Primer-BLAST online tool and verified with the IDT oligo analyzer tool (https://eu.idtdna.com/pages/tools/oligoanalyzer). The primers were synthesized by Invitrogen (Thermo-fisher, USA), and the specific names and sequences of primers used in this thesis project are detailed in tables given below in different sections of methodology.

3.2.3 Genomic DNA extraction

Genomic DNA was extracted from three young leaves from *Petunia x hybrida* using Qiagen DNA Extraction Kit (Qiagen, Haan, Germany) according to the manufacturer's instructions. The Leaf tissues were fragmented with a Tissue Lyser II (Qiagen, Haan, Germany). DNA concentration was measured using a Nano-drop spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA) and diluted to a final concentration of 30 ng μl^{-1} in TE buffer (pH 7.0) for further experiments. The integrity of the genomic DNA was verified by 1% agarose gel electrophoresis prepared by dissolving agarose powder in 1X TAE buffer and adding 1× Sybr® Safe DNA gel stain at the concentration recommended by the manufacturer (Life Technology, Carlsbad, CA, USA).

3.2.4 Sequence amplification

PCR assays were performed for the amplification of the CDS and of the promoter region of *PhMYB106* in a total volume of 20 µl containing 30 ng of genomic DNA template, 1× Platinum Multiplex PCR Master Mix (Applied Bio systems, Carlsbad, CA, United States), GC enhancer (Applied Bio systems), 0.1 µM forward primer (Invitrogen), 0.1 µM reverse primer Invitrogen (**Table 1**) and sterile water to volume. A 9600 Thermal Cycler (Applied Bio systems) was used for PCRs setting the following thermal conditions (**Table 2**). Amplified fragments were subjected to Sanger sequencing to confirm the sequences predicted. The PCR products were visualized on 1 % agarose gel (1% agarose, 1× TAE buffer and 1× Sybr-Safe DNA stain; Life Technologies) placed in the electrophoresis machine at 100 volts for 20 min. Gel was visualized on an UV doc HD6 trans illuminator (UV Tec, Cambridge, United Kingdom) equipped with a digital camera.

Table 1. Primers used for the CDS and promoter region amplification of *PaMYB106* gene in *Petunia hybrida.*

Gene	Primers	Investigation	Sequence (5'>3')	Locus Position
PaMYB106 (Peaxi162Scf03974g00003.1)	I_ex_01_Fw	CDS	ATGGGAAGAATCCCATGTTGCGAAAAAGAG	+1
	III_ex_03_Rev	CDS	TCAAACCATTGGATTCAAGAGATCATCAGATGATA	+1510
	Prom_01_Fw	Promoter	GACTCTAGGAAAGCGAAAATCTGCGG	-1022
	I_ex_01_Rev	Promoter	CTCTTTTTCGCAACATGGGATTCTTCCCAT	+1

***FW** represents forward primer and **Rev** represents reverse primer used.

Table 2. PCR Thermo-cycler conditions used for the amplification of the CDS and promoter region of *PaMYB106* gene.

Stage	Time	Temperature °C	No. of cycles
Denaturation	1 min	94	1 X
Denaturation	30 sec	94	
Annealing	30 sec	55	35 X
Extension	1 min	72	
Extension	10 min	72	1X

3.2.5 Cloning and sequence analysis

Using the TOPO-TA cloning kit (Invitrogen) and following the manufacturer's instructions, all positive PCR-amplified fragments were cloned into the pCR2.1-TOPO vector. The clones were plated on LB/Amp/X-Gal agar, and color-selected colonies were purified using the Qiaprep Spin Mini-prep (Qiagen). Sanger sequencing was carried out using the universal M13 Forward and Reverse primers in order to confirm the cloned nucleotide sequence.

3.3 RNA extraction

Petunia leaves and flower buds were collected from the plant grown in pots according to a classification produced from literature (Kapoor et al., 2002 and Kovaleva et al., 2018) (**Table 3**)

and flower buds were staged at (0.3 cm, 0.5 cm, 0.7 cm, 1.0 cm, or 1.2 cm). The anthers were carefully separated and both anthers and remaining organs of the bud were quickly treated with liquid nitrogen, and stored at -80° C for expression analysis.

Table 3. Different measurements of Petunia flower bud used for RNA extraction.

Bud length (cm)	Morphological event
0.3	PMC development (Meiosis)
0.5	Tetrad formation
0.7	Tapetum formation completed, uni-nucleate microspore development starts
1	Tapetum degeneration
1.2	Tapetum degeneration

3.4 RT- PCR and housekeeper test analysis

The primers selected for q-RT PCR analysis were specific for the *MYB106* gene and for a housekeeping gene (i.e. Elongation Factor 1α) that was appropriate for petunia floral organs. The primer sequences are listed in **Table 4**.

Table 4. Primers used for amplifying the *MYB106* and housekeeper gene in *Petunia x hybrida*

Gene identification	Primers	Sequence 5'>3'	Locus position
PaMYB106 (Peaxi162Scf03974g00003.1)	III_ex_01_Fw	CGGGCCGTACTGACAATGATGTTAAGAACC	+794
	III_ex_02_Rev	TCACTTATCAGGTGAGAGAATGGCTTGTGC	+879
Elongation Factor 1 α subunit (SGN-U207468	EF1α_Fw	CCTGGTCAAATTGGAAACGG	+1090
	EF1α_Rev	CAGATCGCCTGTCAATCTTGG	+1173

***FW** represents forward primer and **Rev** represents reverse primer used

Total RNA was extracted from the anthers and from the remaining tissues of the floral bud without the anther using a RNeasy Plant Kit (Qiagen, Haan, Germany), according to the manufacturer's instructions (three biological replicates consisting of a pool of six buds). Reverse transcription was performed using the Prime-Script™ RT Reagent Kit following the manufacturer's protocol

(Takara, Dalian, China). The subsequent q-RT PCRs were performed in a Thermo-Fisher Quant-Studio 3 real-time PCR instrument (Thermo-Fisher) using SYBR® Green Real-time PCR Master Mix. The assays were performed in 96-well optical PCR plates (Applied Bio-systems, Foster City, CA, U.S.A.), using a Quant-Studio 3 Real-Time PCR system following the experimental conditions suggested by the manufacturer. Conditions set for q-RT PCR are described in the **Table 5** given below.

Table 5. q-RT PCR reaction conditions for *MYB106* and house keeper gene in *Petunia x hybrida*.

No. of cycles	Temperature °C	Time
1	95	20 seconds
40	95	1 second
40	60	20 seconds
1	95	15 seconds
1	60	1 minute
1	95	15 seconds

After analyzing the qPCR machine data, the 2-△△CT technique was used to calculate the differences in gene expression. For that Ct values (average cycle thresholds) of every sample were measured using standards produced by Thermo-Fisher's Quant-Studio Design and Analysis Software v1.4.

3.5 Single guide (SgRNAs) design and in-vitro validation

a) SgRNA design

Using the *PaMYB106* (Peaxi162Scf03974g00003.1) sequence that is available on the Sol-genomics (Sol Genomics Network) platform, the possible regions of interest for mutagenesis induction were tracked in order to generate single guide RNAs (SgRNA). This sequence was used as input to the CRISPOR digital platform (accessible at https://crispor.tefor.net) to identify DNA target sequence ending with a proto-spacer adjacent motif (PAM) sequence, NGG at their 3' end. After comparing the sequences to the Sanger sequencing analysis results, and based on the genome variants, on-target scored SgRNAs were predicted in the CDS and promoter regions of the gene respectively. Three different single guides were designed on the first, second and third exon in the CDS region and three single guides were designed in the promoter region. Perfectly homologous regions were selected for guide RNA design and regions with potential mismatches in the genome

were eliminated from consideration. For in-vitro transcription of SgRNAs, the primers synthesized were obtained from Invitrogen (Thermo-Fisher Scientific, Waltham, MA, USA).

b) Cleavage assay

To perform in-vitro cleavage assay the Guide-it Complete SgRNA kit (Takara, Cat. No. 632636) was utilized to synthesize and confirm the efficacy of the SgRNAs generated by bioinformatics tools. Following are the steps involved in the protocol of in-vitro cleavage assay:

i. Single guide RNA template amplification using PCR

In order to produce a DNA template for the SgRNA in vitro transcription it is important to design a specific primer which includes Guide-it Scaffold template, for the PCR reaction. T7 promoter, SgRNA target sequence and the Guide-it Scaffold template sequence should all be included in this primer. **Table 6** summarizes the list of SgRNA primers used for the CRISPR/Cas9 cleavage assay performed on CDS and promoter region.

Table 6. SgRNA primer list and other related information. Locus position: position relative to the predicted ATG. Length of pre amplified DNA target before in vitro cleavage. Expected length of fragments produced due to cleavage assay.

Primer name	Sequence 5'>3'	PAM	Locus position	Amplified DNA target (bp)	Cleaved fragment length (bp)
SgRNA_Prom_01	AGAATTCAGGCGATCTCACG	AGG	-521	1100	520+580
SgRNA_Prom_02	TAGCTCACCCACAAGACACC	CGG	-346	1000	350+650
SgRNA_Prom_03	AGAGGTATGTCGAAAGGCAT	GGG	-243	1100	300+800
SgRNA_Iex	GAGGGGGCAATGGACTCCTG	AGG	34	870	130+740
SgRNA_IIex	CTCCGGCCTGATCTCAAACA	TGG	252	820	260+560
SgRNA_IIIex	ACTGGAAGTACGTGCACAGT	AGG	1507	800	520+280

PCR reaction was carried out in accordance with the manufacturer's procedure, using a Prime STAR Max Premix DNA polymerase. In accordance with the protocol, the reaction occurs in a final volume of 25 µl under the following conditions; 98°C for 10 seconds and 68°C for 10 seconds for 33 consecutive cycles. This yields an accurate and highly concentrated RNA template. After PCR was completed and to confirm that the sg RNA template was amplified, 5 µl of the PCR product was examined on a 2% agarose gel.

ii. In vitro transcription of Sg RNA

For in vitro transcription of SgRNA, it was necessary to use 5 µl of the prior PCR product as a template for sg RNA synthesis with the IVT kit using Guide-it T7 polymerase mix and Guide-it Transcription Buffer in order to perform this step. Then PCR tubes were incubated for the synthesis reaction at 37 °C for minimum 4 hours. After the incubation, it was mandatory to remove the template DNA which was done by adding Recombinant DNase I to the reaction mixture, followed by an additional incubation for 15 minutes, as a result SgRNA containing the target sequence was generated.

iii. Purification of transcribed SgRNA

The purification of transcribed SgRNA was done after performing the digestion with recombinant DNase I (RNase-free) using the Guide-it IVT RNA Cleanup Kit. The concentration of purified and transcribed SgRNA was determined through a Nano-Drop 2000 spectrophotometer.

iv. DNA amplification of target gene region

The targeted (CDS and promoter) region of our putative gene *PaMYB106* were re-amplified after bioinformatics analysis, confirmed the existence of potential SgRNAs. These regions served as a reference for the in vitro cleavage assay reaction since they have already been cloned, amplified, and sequenced.

v. In vitro cleavage assay

Following SgRNA purification, 1 µl of each SgRNAs (50 ng/µl) was combined with 0.5 µl of Cas9 nuclease (500 ng/µl) and incubated at 37 °C for 5 min to create the RNP assembly. Subsequently, the RNP assembly was supplemented with 1 µl of BSA, 1 µl of 15X Cas9 Reaction Buffer, 6.5 µl of RNase Free Water, and 250 ng of the target gene PCR product. After carefully mixing all the

components with a pipette, they were immediately incubated for 1 hour at 37 °C and then for five minutes at 80 °C. Using the supplied Guide-it recombinant Cas9 nuclease and the purified SgRNAs an excision reaction was carried out on the DNA templates as part of the in vitro restriction.

vi. Examination of the excision product

Following the incubation, the in vitro digested products were evaluated using 1.5% agarose gel electrophoresis

3.6 Isolation and purification of protoplast

Protoplast isolation

The protoplast isolation procedure was carried out in accordance with (Nicolia et al., 2021 and Tan et al., 1987) standards, based on the protocol already used for tomato. Some specific modifications based on the framework of (Kang et al., 2023) were made for petunia.

a) Preparation of Solutions

All stock solutions were prepared using double distilled water (ddH2O) and filtered through 0.22 µm syringe unless otherwise specified. The main buffers and solutions with their final concentrations used for protoplast isolation are listed in the tables given below

Table 7. *Digestion/ Cell lysis buffer:* List of chemicals and enzymes with their final concentrations used to prepare digestion buffer.

Chemical/Enzyme	Final concentration
D-Mannitol	0.4 M
MES	20 mM
KCl	20 mM
CaCl2	10mM
Macerozyme	0.5%
Cellulase	1%
Pectolase	0.05%
BSA	0.1%

*The pH was adjusted to 5.8 using 1 N KOH.

Table 8. *Washing buffer (W5)*: List of chemicals and their final concentrations used to prepare W5 buffer

Chemical	Final concentration
MES	2 mM
KCl	5 mM
NaCl	154 mM
CaCl2	125 mM
Glucose	5 mM

*The pH was adjusted to 5.8 using 1 N KOH

Table 9. *Cell and Protoplast Washing solution (CPW):* List of chemicals and their final concentrations used to prepare CPW solution

Chemical	Final concentration
MES	2 mM
CaCl2	10 mM
KH2PO4	0.2 mM
KNO3	1 mM

MgSO4*7H2O	1 mM
KI	0.96 µM
CuSO4*5H2O	0.16 µM
D-Mannitol	11%

Table 10. *MMG buffer:* List of chemicals and their final concentrations used to prepare MMG buffer.

Chemical	Final concentration
D-Mannitol	0.5 M
MES	4 mM
MgCl2	15 mM

* The pH was adjusted to 5.8 using 1 N KOH

Table 11. *PEG Calcium Transfection Media*: List of chemicals and their final concentrations used to prepare transfection media.

Chemical	Final concentration
PEG 4000	40% (w/v)
D-Mannitol	0.2 M
CaCl2	100 mM

* The pH was adjusted to 5.8 using 1 N KOH

b) Preparation and isolation of Petunia protoplasts

Young leaves weighing 0.6 grams were taken from 4 weeks old in vitro grown *Petunia x hybrida* hort. ex E. Vilm plant, and were placed in a Petri dish containing 15 ml of cell lysis buffer (details of buffer composition mentioned in Table 6). Using a sterile surgical blade, leaves were sliced into smaller strips in the digestion solution, without damaging the leaf tissues. Extracted leaf material were then incubated overnight in the dark at 25°C with gentle shaking at 20 rpm. After carefully mixing and diluting the protoplast suspension with an equal volume of W5 solution, the mixture was filtered to eliminate any remaining undigested leaf tissues. Then the mixture was slowly filtered through a sieve of 60 µm pore size and pelleted by centrifugation at 100 G for 5 min. The supernatant was carefully removed and pellet was slowly re-suspended in 5 ml of W5 buffer. Protoplast were gently plated on filtered 21% sucrose in CPW solution and centrifuged at 100 G

for 5 min. Protoplast floating on the sucrose layer were collected, washed in 10 ml of W5 buffer and subjected to centrifugation at 100 G for 5 min. The protoplasts were then re-suspended in 5 ml W5 and centrifuged again at 100 G for 5 min and the resulting pellet was again suspended in 200 µl of MMG solution.

c) Determination of yield and viability

The protoplasts isolated from Petunia plant were determined by putting 10 µl of protoplast solution in a Burker-chamber. The counting of the number of protoplasts was done under the microscope at 40X resolution. Only intact protoplasts were counted in 12 different chamber cells. Protoplast density was calculated as follows:

$$\text{Protoplast no. } g^{-1}\text{-FW leaf} = \text{Avg. protoplast no. per chamber cells} \times \mu\text{l protoplast suspension} \times \frac{\text{g FW leaf}}{104 \times 250}$$

The viability of protoplasts was determined by adding 2 µl Fluorescein Diacetate (FDA) staining dye prior to 100 µl protoplast suspension. The solution was placed in the dark for 2-5 min. The yield of viable protoplasts was determined with a fluorescence microscope using as described above for the protoplast total yield. Viability was calculated as the ratio between viable and total protoplast yield.

d) Protoplast transfection

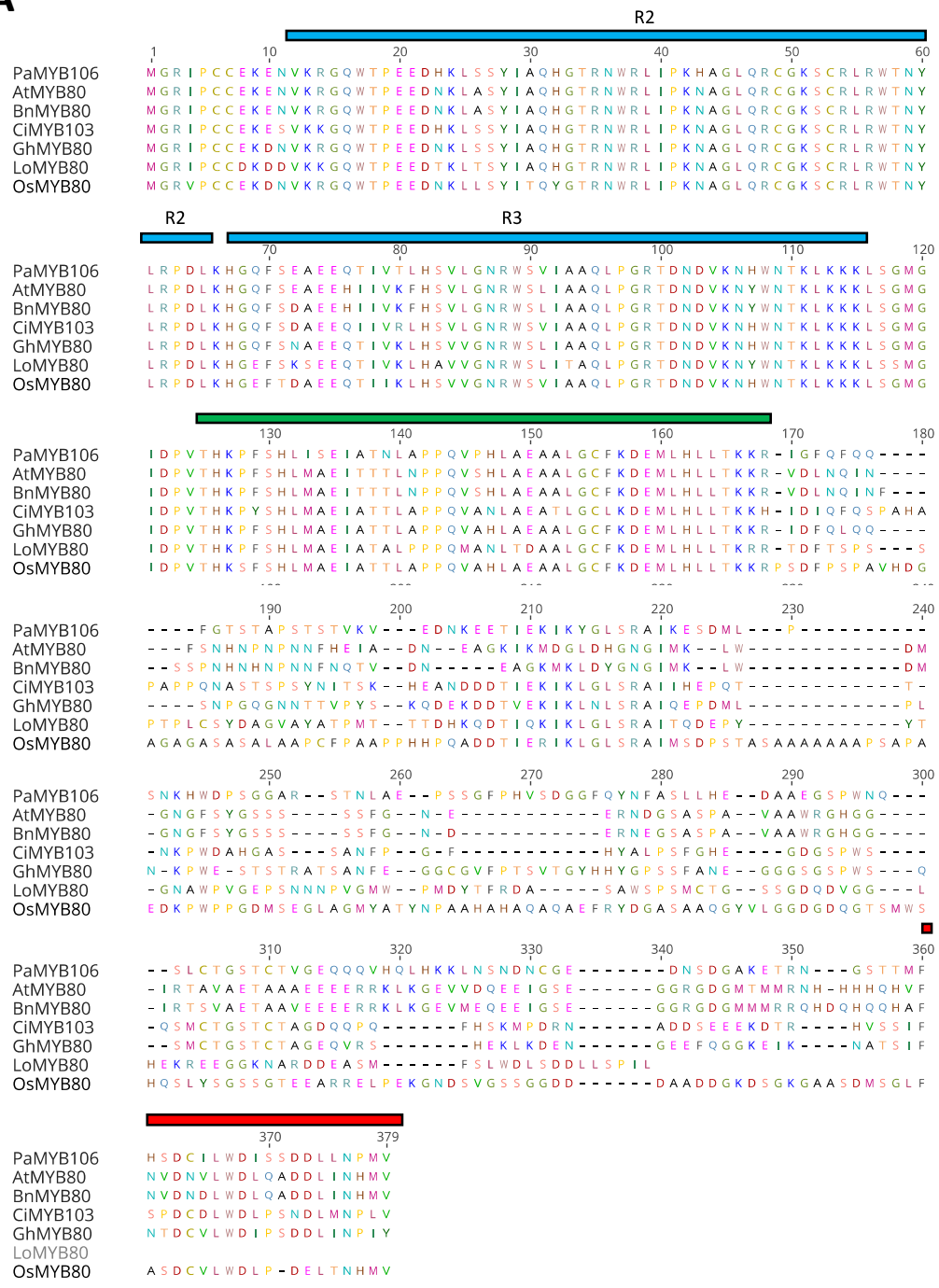
Freshly isolated protoplasts from Petunia leaves were transfected for the transient expression of Green Fluorescence Protein (GFP), following a procedure adapted from the protocol of (Yuan et al., 2023) for proportion of reagents for transfection, with some modifications according to (Kang et al., 2023) were made for Petunia. Protoplast suspension was kept at 4 °C for 1 hour after isolation to stabilize the cells and then diluted in order to have 2×10^5 protoplast suspended in 200 µl of MMG. The solution was mixed with 15 µg of Cas9+GFP fusion protein (Sigma-Aldrich) and 220 µl of freshly prepared 40% PEG 4000 solution before incubating in the dark at room temperature for 15 min. Subsequently, protoplasts were washed twice with 880 µl of W5 solution. After centrifugation at 100 G for 5 min protoplasts were re-suspended in 1 ml of W1 solution and kept in the dark at room temperature. In order to determine the transfection efficiency, the transfected protoplasts were visualized under fluorescence microscope, following the procedure already described for the protoplast viability measurement, after 15 min and after 24 hours.

4. RESULTS AND DISCUSSION

4.1 Bioinformatics analysis

Preliminary bioinformatics analyses were carried out in order to evaluate whether the *PeaxMYB106* sequence can be a candidate gene for the induction of male sterility in Petunia. Multiple alignments were performed between the amino acid sequence of interest and six other homologous genes, previously characterized and linked to the onset of male sterility in other plant species, including *A. thaliana*, *O. sativa* and *G. hirsutum* (**Figure 3.A**) and a phylogenetic tree was created on the bases of sequence diversity (**Figure 3.B**). Although a long phylogenetic distance between the species was observed (similarity degree between 48.3% and 89.5%), the homologous proteins showed similar lengths (ranging from 320 to 372 amino acids). It is interesting to note that a significant conservation was observed among the first 115 amino acids from all the species, corresponding to the R2R3 MYB domain. R2R3 domain is a DNA binding domain responsible for the transcription factor function (Dubos et al., 2010). The region of 44 amino acids subsequent to the MYB domain also showed a similar pattern among all the sequences analyzed, with peptide conservation stopping at position 169 KKR. In addition, a highly dissimilar region of 149 amino acids was present in all the species, followed by 20 amino acids in the C region, which slightly differed from sequence to sequence (Xu et al. 2014 and Phan et al. 2011) reported that the highly conserved R2R3 DNA-binding domains, the 44 amino acids downstream of the R3 region and the variable C-terminal portion showed by the *PeaxMYB106* orthologs are unique among MYB genes and the interaction between the action of these regions can be critical for the gene function. In fact, (Phan et al. 2012), demonstrated that the addition of an EAR motif in the 44 amino acid region, affected the DNA binding or protein-protein interaction, leading to a non-functional gene. In addition, (Li et al. 2007), observed that a T-DNA insertion that modifies the 18 amino acids in the C region truncates *AtMYB80* and cause MS. In the same context, (Palumbo *et al.* 2019), discovered a 4 bp insertion in the second exon of male sterile plants, responsible for the introduction of a pre-stop codon resulting in a shorter protein of 123 amino acids, which can putatively be non-functional and lead to MS.

A


R2
R2
R3
PaMYB106
AtMYB80
BnMYB80
CiMYB103
GhMYB80
LoMYB80
OsMYB80

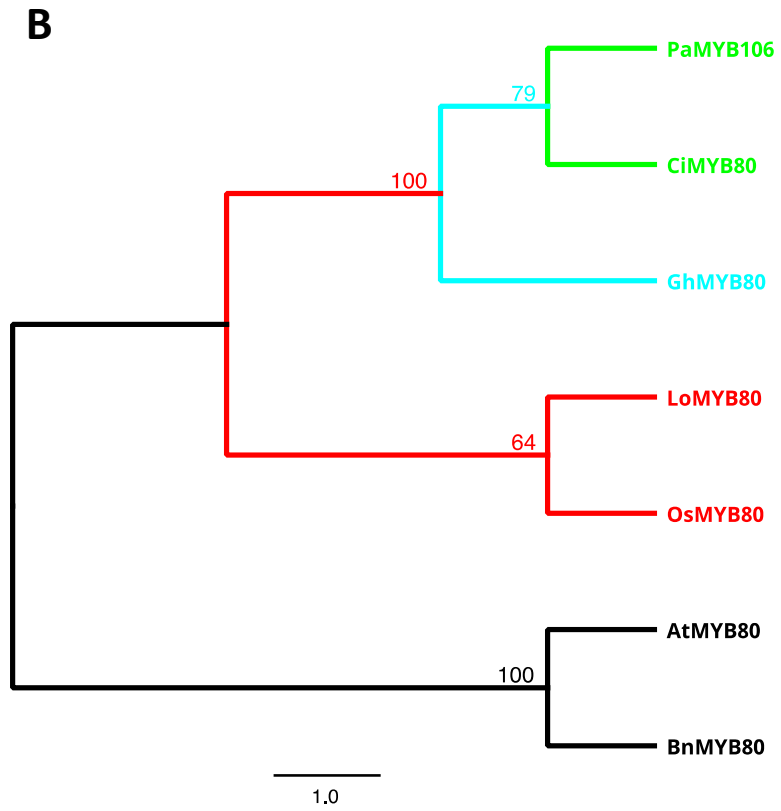


Figure 3. A. Multiple alignment of peptide sequences between petunia *MYB106* and six different MYB potential orthologues was performed using Geneious Prime® software. Aligned peptide sequences from Arabidopsis (*AtMYB80*), canola (*BnMYB80*), chicory (*CiMYB103*), cotton (*GhMYB80*), lily (*LoMYB80*) and rice (*OsMYB80*) plants. The conserved regions are highlighted: The R2R3 domain is indicated by blue boxes, the conserved 44 amino acid sequence region is indicated by a green box, and the C-terminal region is indicated by a red box. **B.** Jukes-Cantor Neighbors Joining tree based on the described alignment.

4.2 *PaMYB106* isolation in *Petunia x hybrida*

Exploiting the genomic DNA isolated from clonal *Petunia x hybrida* plants, propagated under *in vitro* conditions, the full-length CDS of *PhMYB106* was amplified by PCR with the primer pair I_ex_01_Fw and III_ex_03_Rev. The nucleotide sequence identity with the *PaMYB106* sequence retrieved from *P. axillaris* (Lam.) draft genome (available in the Sol Genomics Network database) was confirmed with Sanger sequencing. In silico analysis, generated by the GSDS online tool of gene locus, confirmed the exon/intron organization of the gene structure, which resulted to be composed by three exons (respectively formed by 133, 130 and 739 bp) and two introns (75 and

433 bp in length) (**Figure 4**). Subsequently, the exon–intron organization was confirmed through amplification of a cDNA sequence corresponding to a full-length CDS transcript encoding a putative 334 aa peptides. In addition, a genomic region that was 1022 bp upstream of the ATG site was amplified with the primer pair Prom_01_Fw and I_ex_01_Rev to map the putative TSS and TATA box in the *MYB106* locus through the Soft berry-TSSP software. The predicted TSS region with the most likelihood resulted to be at 56 bp upstream the ATG, while the TATA box at 72 bp. Analyzing the promoter is of relevance for MYB genes, since if they are truncated, promoter represses its action, showing a negative autoregulation mechanism in the later stages of pollen development (Phan et al. 2011; Xu et al. 2014).

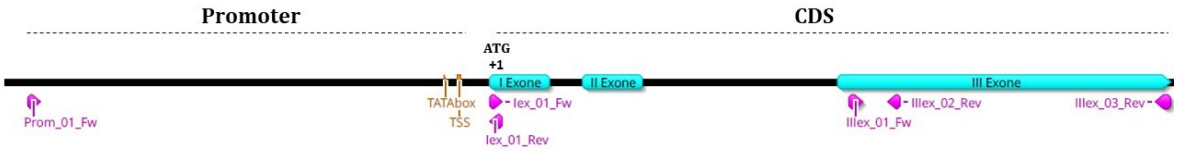


Figure 4. Schematic representation of the *PhMYB106* genomic locus, including the upstream basal promoter and CDS. The relative positions and orientations of the primers are indicated with purple arrows. The predicted TATA-box and TSS motifs in the promoter region are indicated. The exons are indicated with filled light blue rectangles, while the introns are indicated with black lines in the CDS region.

4.3 Expression Analysis

As the putative orthologous *AtMYB80* gene is expressed during anther development, the expression of *PhMYB106* was evaluated in floral buds that were collected at different growth stages (**Figure 5.A**). The results obtained with Real Time RT-PCR showed an almost absent RNA levels in the leaves, while in the flower buds *PhMYB106* was considerably more expressed (**Figure 5.B**). In particular, in anthers the gene expression was higher than in the rest of the flower buds (FBs) and in both anthers and FBs the maximal RNA levels were seen at the lower flower bud size sampled (0,3 cm of height), while the minimal were observed at the highest bud size (1,2 cm). Therefore, in general the expression tended to decrease with the increasing of the bud size, even if an increase at 0,7 cm of bud size for anthers observed. This last observation might be due to fluctuations in the actual decreasing of expression. The highest *PhMYB106* expression coincided with the early

phases of micro-sporogenesis in Petunia, while the decrease is comprehended towards the following micro-gametogenesis phase (Kapoor et al., 2002; Kovaleva et al., 2018). Tapetum reaches the pick of growth of its development when the flower buds are around 0,5 cm of height, while completely ends its formation and starts to degenerate around 1,0 cm. Tapetum is known to be fundamental for viable pollen production, secreting essential nutrients like lipids and polysaccharides which allow pollen maturation (Gómez et al., 2015; Liu & Fan, 2013). Nutrient secretion is determined by specific timing in tapetum degradation, while it was observed in several species that abnormal development of its structure leads to lack of or un functional pollen formation, causing MS (Ito et al., 2007; Parish & Li, 2010). *PhMYB106* orthologs in other species have been reported to be related with correct tapetum and pollen development (Sui et al., 2015; Pan et al., 2020). Coinciding the maximal gene expression with the phase of major tapetum growth and the lowering trend with the phase of its complete maturation, data obtained support the idea of an involvement of *PhMYB106* in the functional tapetum/pollen development also in Petunia and therefore the gene of interest can be a potential target for MS induction.

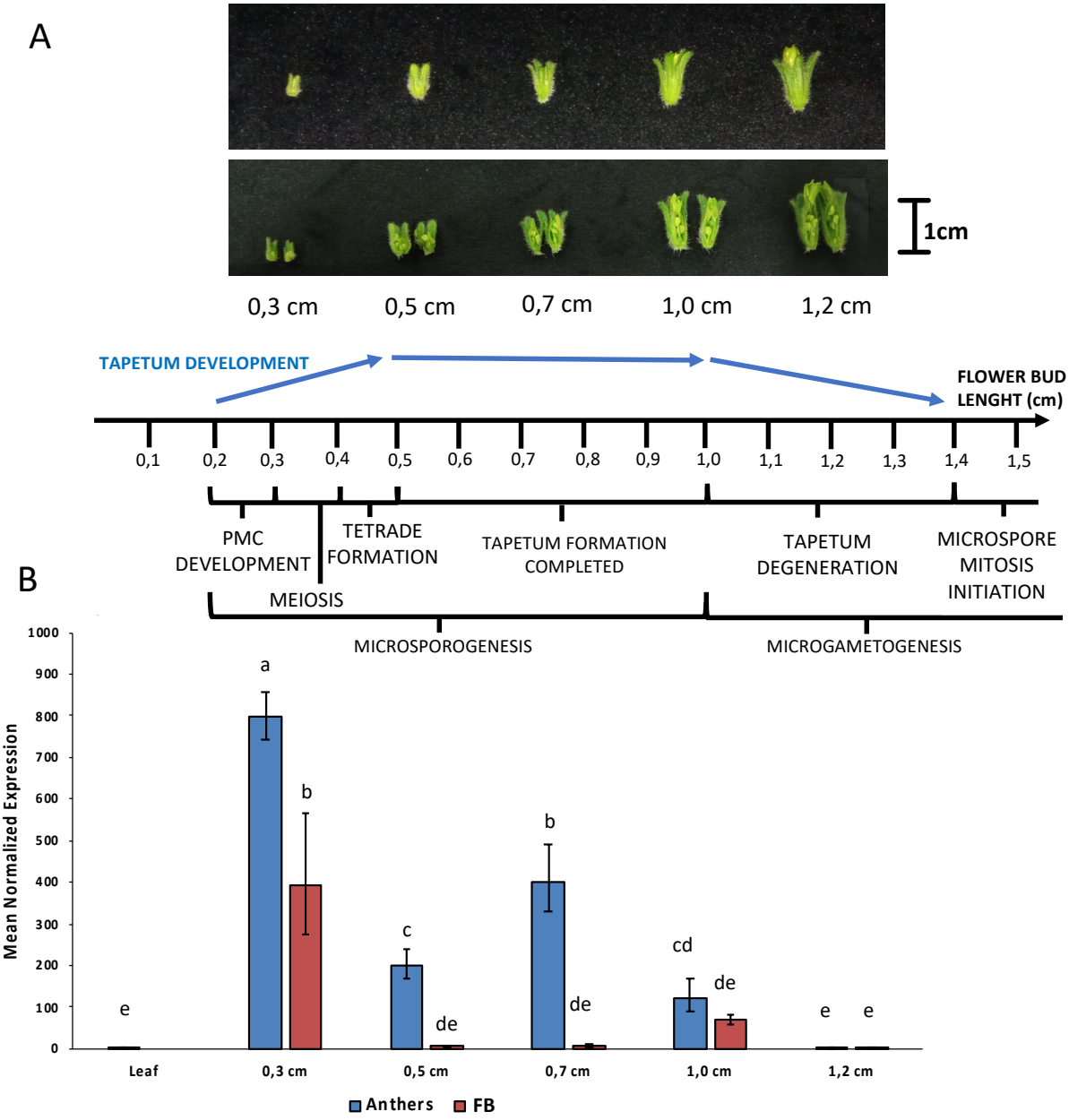


Figure 5. *PhMYB106* relative expression analysis. **A.** Flower buds and flower buds sections, staged and collected at different growth stages, with the relative development stage of Petunia pollen structures. **B.** Normalized expression level of the *PhMYB106* transcript as measured by real-time q-RT PCR. The data were analyzed to determine gene expression using the $2^{-\triangle\triangle CT}$ method, in comparison to the expression of the housekeeper gene Elongation Factor 1α and of *MYB106* gene in leaf. Error bars represent the standard deviation. Lowercase letters represent significant

differences at $p<0.05$ according to one-way ANOVA (Tukey HSD test). Blue bars: Anthers. Red bars: Flower Buds without anthers (FB).

4.4 SgRNA design and *in vitro* cleavage assay with CRISPR-Cas9 system

Preliminary study of the sequence of the *PhMYB106* genomic locus was fundamental to support the design of specific guide RNAs (SgRNAs), in order to find specific regions suited for becoming targets for generating CRISPR/Cas-edited DNA via direct delivery of an in vitro preassembled ribonucleoprotein (RNP) complex, formed of the Cas9 endonuclease and selected SgRNAs. Through the use of the CRISPOR2 platform, several candidate SgRNA binding sites in the *MYB106* locus were found, including both in CDS and promoter region. Regarding the CDS, attention was given to the selection of SgRNAs that target each of the three identified exons, while, for promoter, guides located around the predicted TATA-box motif were chosen. As demonstrated for other orthologous MYB genes, the editing of coding sequences, as well as of regulatory regions, could be an attractive strategy to block the production of a functional protein at the pre- or posttranscriptional level (Phan et al. 2011; Xu et al. 2014).

Among the CRISPOR outputs obtained, six SgRNAs were selected as potential effectors on the basis of the recognition site and the minimum number of predicted off-targets. These potential SgRNAs were in vitro synthetized, and the correct Cas9-SgRNA assembly was subsequently confirmed: a correct cleavage by the Cas9 protein complex was observed in the amplified region, comprehending the sequence recognized by the SgRNA. The lengths of the pre-amplified targets, including the DNA fragments that are recognized by the S gRNAs, are shown in **Table 2** and in **Figure 5A**. The results after in vitro cleavage assay with the Cas9/SgRNA_Prom02 and Cas9/SgRNA_IIex complexes are shown in **Figure 5B.** Fragment lengths obtained corresponds to those predicted, confirming the suitability of the SgRNA selected for CRISPR-Cas9 system applications.

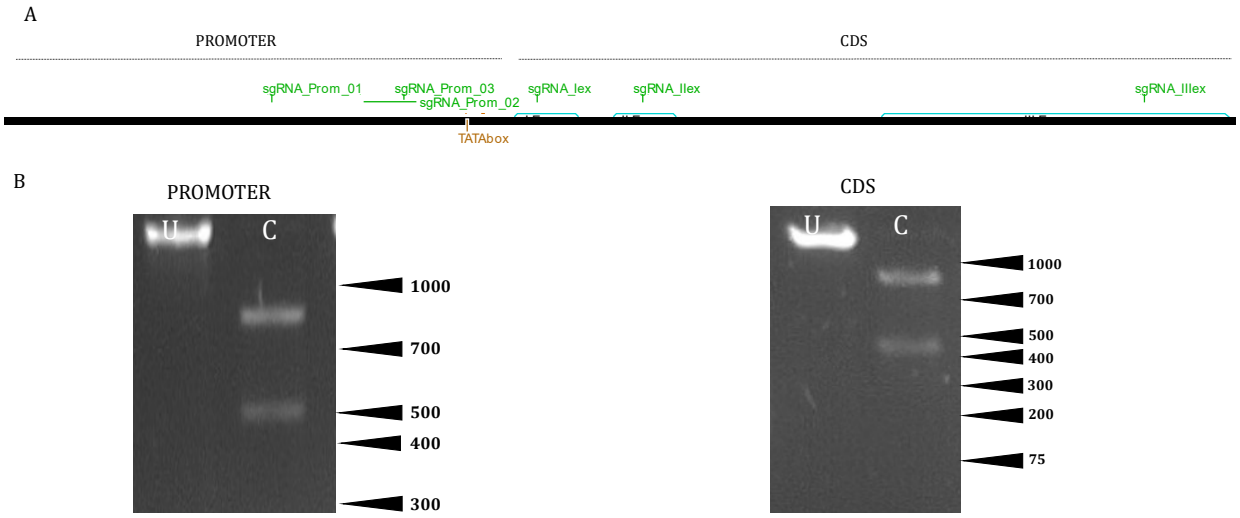


Figure 5. Design and in vitro cleavage assay of SgRNAs. **A)** Schematic representation of the *PhMYB106* genomic locus, including the upstream basal promoter and CDS region. The mapping of all six SgRNAs is visualized in green. **B)** In vitro Cas9 cleavage assay using SgRNA_Prom02 and SgRNA_IIex for the promoter and second exon, respectively. U: un-cleaved; C: cleaved. Agarose gel (1%). The molecular marker used was the Gene Ruler 1Kb Plus DNA ladder (Thermo-Fisher).

4.5 Protoplast isolation and transfection

Protoplasts are the plant cells that lack a cell wall and maintain their genetic information, allowing for the rapid insertion of desired genes. This trait makes them useful for genetic research and modification. As described in the introduction section, CRISPR/Cas9 construct can be delivered into the cells in the form of RNP complex, thus avoiding the integration of foreign DNA providing a more efficient and precise means of gene editing.

For isolating the protoplasts from leaves of *Petunia x hybrida* hort. ex E. Vilm the protocol adapted was based on the one already used for tomato with slight modifications according to Kim et al., 2022 were made only for the transfection. The conditions which results in intact shaped, high yield and viable protoplasts are; in vitro growth conditions which includes sterile plant material grown on MS medium, enzymolysis process which includes 0.4 M osmotic concentration and overnight incubation in dark with shaking at 20 rpm. For purification, 21% sucrose and centrifugation speed

(100 G for 5 min) was a critical point for obtaining viable protoplasts. **Figure 6**, schematically represents different steps followed during protoplast isolation and purification procedure.

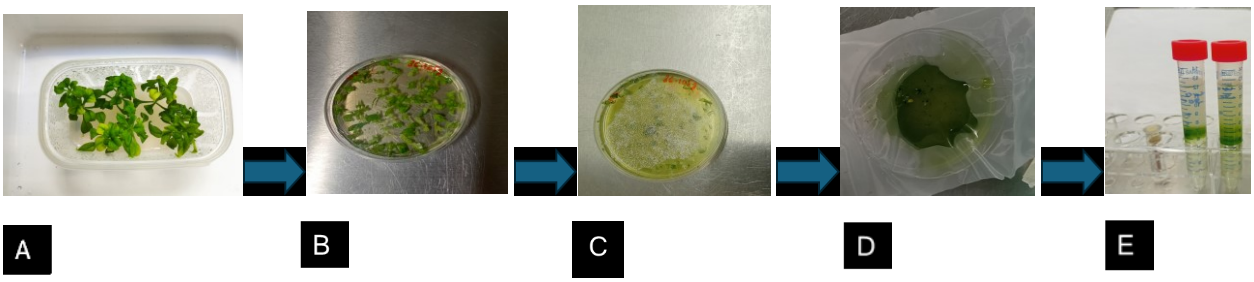


Figure 6. Summary of the stages involved in isolating protoplasts from in vitro cultivated *Petunia x hybrida.* **A**) 4-5 weeks old Petunia plant grow in vitro. **B**) Petunia leaves chopped into 0.3 mm segment in lysis buffer. **C**) Enzymatic digestion of Petunia leaves after overnight incubation at 20 rpm. **D**) Enzymatic solution containing protoplasts filtered through a 60 µm nylon mesh into a sterile Petri dish. **E**) Protoplast ring formed due to sucrose gradient after centrifugation.

In literature different conditions are used for protoplast isolation specifically for Petunia, however both tomato and Petunia belongs to same plant family therefore we adapted those conditions which were already used for tomato. The important step during protoplast isolation and purification is the formation of protoplast ring produced due to sucrose present in CPW solution as we can see in **Figure 6** (**E**), and we obtained a thick layer of ring indicating high amount of protoplast. The following steps require careful handling since these protoplasts are very delicate because they lack cell wall.

Yield and Viability

Controlling yield throughout the isolation process is crucial for achieving the desired shape, vitality, and density in our protoplasts. When the desired levels of quality and quantity for isolation are met, it is assumed that we have the sufficient and good starting material for transfection. **Figure 7** shows some images of freshly isolated protoplasts visualized under microscope at 10X and 40X resolution.

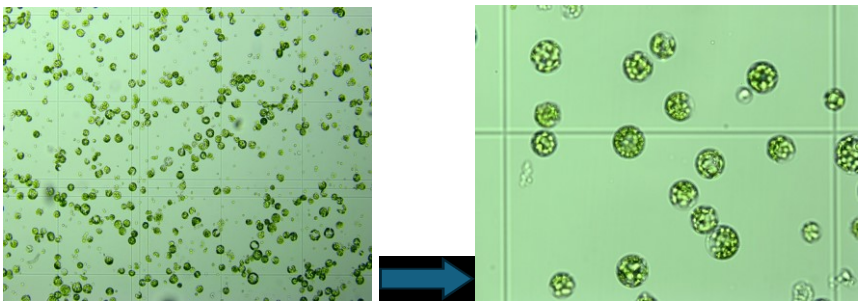


Figure 7. Freshly isolated Petunia protoplasts under microscope at 10 and 40X resolution

Protoplast density is critical for transfection. With this optimized protocol we obtained the density of 2×10^5 from 4 weeks old in vitro grown Petunia plant. In **Figure 7** we can observe the intact shape of protoplasts, after obtaining these results the next step was to check viability. For transfection it is necessary to have viable protoplasts, therefore we determined the viability of protoplasts by using FDA and results were recorded. **Figure 8** represents optimal yield of viable protoplasts obtained. Viability was measured using FDA staining method before transfection. Images were taken under fluorescence microscope using different filters such as Bright field (BF), FDA and merged at 10X.

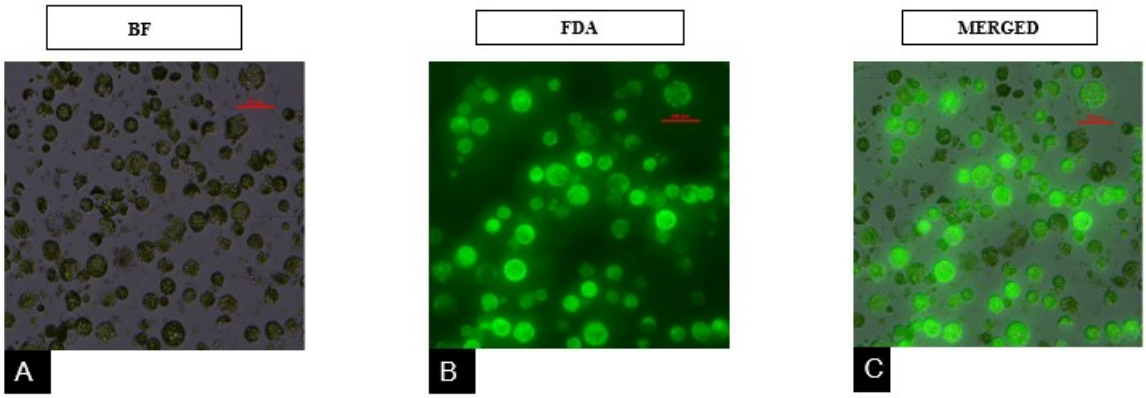


Figure 8. Optimal yield of viable protoplast obtained after FDA treatment. A) Protoplast under bright field (BF). B) Viable protoplast under FDA filter. C) Merged image of BF and FDA showing alive and dead protoplast. Scale used is 100 µm.

Protoplasts were tested for quality during isolation based on their circular form, indicating cell wall digestion and viability under a microscope. The accurate isolation of protoplasts allowed us

to proceed to the transfection step, which is an important for the delivery of RNP complex and DNA-free CRISPR-Cas9 transient expression.

Protoplast transfection

In order to examine transient expression system, the modified PEG mediated protoplast transfection protocol based on the method described by (Kang et al., 2023). To optimize GFP expression in Petunia leaf protoplasts, we transfected them using a GFP-Cas9 fusion protein. In this step we examined the amount of GFP-Cas9 fusion protein, PEG concentration and transfection time, to obtain the results previously reported by (Kang et al., 2023). However, it was observed that 15 µg of Cas9-GFP fusion protein, 40% PEG and 15 min for transfection yield positive results and a consistent signal of RNP complex was obtained in successfully transfected protoplasts when closely examined under fluorescence microscope. In **Figure 9** we can observed a successful transfection of protoplast with Cas9-GFP fusion protein. This result was obtained after 15 min of transfection. The efficiency of transient expression is strongly influenced by several factors, which we have already discussed and taken into account. However, it is fundamental that protoplast remains viable and transfected after 24 hours or even more in order to proceed with regeneration step in future. In this experiment we observed transfection efficiency and viability after 15 min and 24 hours and results were recorded. **Figure 10** shows transfected protoplasts after 24 hour examined under microscope.

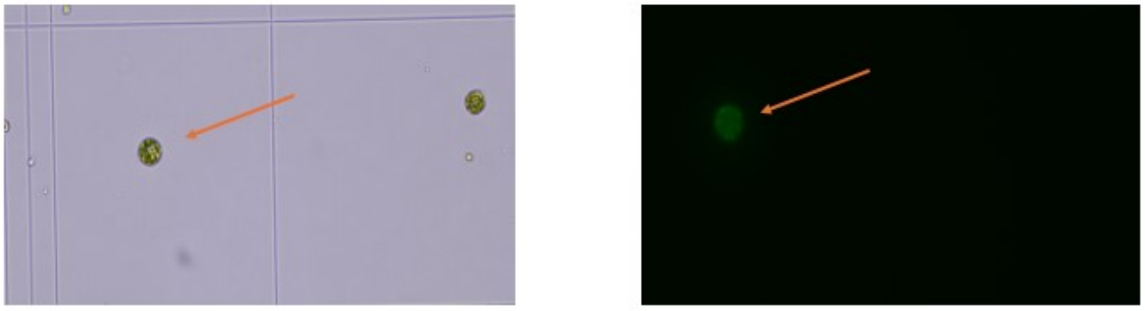


Figure 9. Petunia protoplasts transfected with a Cas9 -GFP protein for transient expression. 40% PEG was used for transfection. Results obtained after 15 min of transfection. Arrows indicate transfected protoplast under BF and GFP filter.

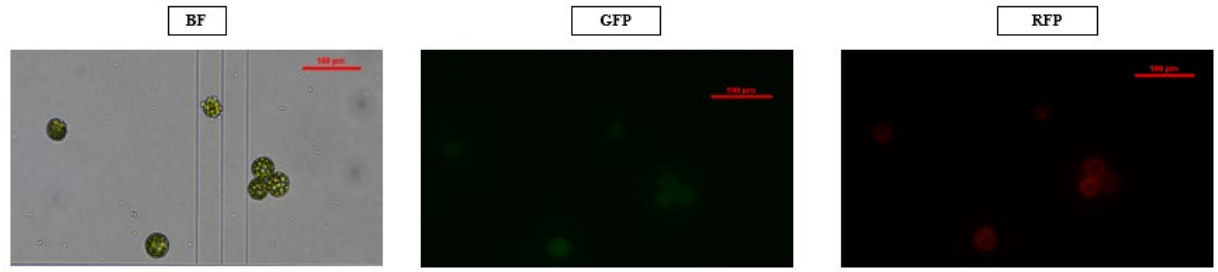


Figure 10. Petunia protoplast transient expression with Cas9-GFP fusion protein after 24-hour transfection. Images taken under fluorescence microscope, with different filters BF, GFP and RFP at 40X magnification. Scale used is 100 µm.

Conclusion and future perspectives

This study focuses on the establishment of the beginning steps for establishing a CRISPR/Cas9-based breeding strategy for implementing the MS trait in *Petunia x hybrida* hort. ex E. Vilm, a model system for the Solanaceae family. This research aims to introduce a new tool for plant breeding that can yield MS lines, complementing traditional methods. Based on the results obtained from the alignments of *PeaxMYB106* with previously confirmed putative orthologs revealed the presence of functional domains which are common among the other MYB proteins and the interaction between the action of these regions can be critical for the gene function.
The expression analysis of *PhMYB106* carried out in this study and the results obtained revealed that *PhMYB106* transcription factor gene may have an involvement in post-meiotic phases of development, specifically in functional tapetum/pollen development in Petunia, as orthologs in Arabidopsis, cotton, and rice, which have been reported to be related with correct tapetum and pollen formation. This led to speculate on the potential role in MS induction in Petunia. For this purpose, CRISPR/Cas9 gene editing technology was the most suitable method for testing our theory. This system has proven to be an ideal GE tool for inducing specific mutations in various plant species and effective in generating sterile lines than conventional breeding. However, RNP-DNA-free modification of genes involved in anther/pollen development could be a viable alternative to the traditional use of CRISPR/Cas9 technology.

In this study, a temporary protoplast transfection system that is mediated by CRISPR/Cas9 edited DNA-free plant material was created in Petunia. To successfully apply this system, several elements were considered, including SgRNAs designed using digital platforms in the targeted locations of *PaMYB106* as well as RNP complexes and delivery conditions. However, after successfully demonstrating the RNP complex structure in the in vitro experiments, we proceed with the protoplast isolation and transfection protocol which previously yield satisfactory results. However, we successfully obtained the protoplast transfection results and method used was reproducible, providing a basis to establish a preliminary regeneration protocol for *Petunia x hybrida* in future. Despite that the transfection efficiency was lower compared to that reported in the literature, primarily due to factors discussed in the previous section. Therefore, further investigation is required to address this gap. In the future, after obtaining a high efficiency of transfection in protoplasts, it is possible to evaluate the functionality of RNP complex in vivo. For

this purpose, we can exploit the totipotent nature of protoplasmic cells to regenerate into modified plants in vitro. Later, we can perform sequencing analysis to identify genetic differences in potential mutants, as well as histological and expression analysis to confirm the significance of the *PaMYB106* gene.

The main objective of this research aligns with the European goal to achieve and promote sustainability. Gene edited plants are typically indistinguishable from the naturally occurring mutants. Moreover, off-target effects are minimal in DNA-free approaches, unlike other stable and transient expression methods. However, it is supposed that Cas9 RNPs cleave target DNA immediately after transfection, degrades rapidly within hours limiting their mode of action to the original cells (protoplasts) of the edited plants. However, Europe focuses on the generation process during the approval of new plant products. Therefore, DNA-free genome edited plants will be subjected to the same scrutiny process as the few legally cultivated genetically modified plants in Europe. This will ultimately benefit the trade business of ornamental plants, since many countries have now started to update their legal interpretations related to the modified plants.

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