Pollen polymorphism in Magnoliaceae and conservation of plant male germline regulators

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Abstract

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In flowering plants twin sperm cells are produced by the male gametophyte to enable double fertilization, which is of vital importance for plant fertility. The process of male gametogenesis depends upon relatively poorly understood mechanisms of gamete cell production, but the male germline-specific R2R3 MYB transcription factor DUO1 is an essential factor in germ cell division and gamete differentiation in *Arabidopsis*. One major objective of the thesis was to assess the validity of Brewbaker's evidence that Magnoliaceae possess exclusively bicellular pollen at anthesis. This analysis provided conclusive evidence of the coexistence of polymorphic (bicellular and tricellular) pollen in Magnoliaceae species. Further, the maturation of pollen under increased temperature regimes can significantly accelerate the rate of generative cell division in *M. grandiflora*. A second major aim was to identify sequence and functional conservation of *DUO1* orthologs genes in angiosperms and bryophytes. This study provided further evidence that a supernumerary lysine residue in the DUO1 MYB domain is widely conserved and the sequences and expression of two DUO1 orthologs from moss (*Physcomitrella patens*) were validated. *Arabidopsis*, tomato and rice DUO1 orthologs were shown to transactivate *Arabidopsis* DUO1 target promoters. The expression of angiosperm DUO1 orthologs were able to complement the generative cell division and genetic transmission defects in *duo1* mutants, demonstrating conservation of function of DUO1 orthologs in *Arabidopsis.* The final objective was to investigate the functional conservation of DAZ1/DAZ2–related proteins in other angiosperms. The *AtDAZ1* orthologs from *Brassica rapa* (*BrDAZ1*) and tomato (*SlDAZ1*) were able to partially rescue the division and transmission defects of *daz1/daz2* double mutant pollen, allowing *daz1/daz2* homozygous lines to be established harbouring heterologous DAZ1 transgenes. Collectively, these results show conserved functions for both DUO1 and DAZ1/DAZ2 orthologs, highlighting the important role of the transcriptional network controlled by these male germline-specific regulators in from angiosperms.

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Dedication

This thesis is dedicated to my brilliant and beautiful fiancé ESRA who is my soul mate, for her love, endless support, and encouragement.

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Abbreviations

BAP	6-benzylaminopurine
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
^{o}C	Degrees centigrade
CDKA;1	Cyclin-dependent kinase A;1
cDNA	Complementary DNA
chi	Chi-square statistic
cm	Centimetre
CoA	Coenzyme A
CDK	Cyclin-dependent kinase
CDS	Coding sequence
DIC	Differential interference contrast
DAPI	4',6-diamidino-2 phenylindole dihydrochloride
DAT	DUO1-activated target
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DUO1	Duo Pollen-1
DUO2	Duo Pollen-2
DUO3	Duo Pollen-3
EAR motif	ERF-associated amphiphillic repression motif
EDTA	ethylenediaminetetraacetic acid
F1	First generation after a cross
Fluc/Rluc	Normalised dual luciferase activity
fm	fentamole
g	Gram
G	Guanine
gDNA	Genomic DNA
GEX2	Gamete expressed-2
GFP	Green fluorescent protein

GUS	Beta-glucuronidase
H2B	Histone H2B
kb	kilobase pair
l	Litre
fLUC	Firefly luciferase protein
M	Molar
MES	2-(N-Morpholino)ethanesulfonic acid
mg	Milligram
MGH3	Male gamete-specific histone-3
ml	Millilitre
mM	Millimolar
mRFP	Monomeric red fluorescent protein 1
mRNA	Messenger RNA
MS	Murashige and Skoog
ng	Nano gram
NLS	Nuclear localisation signal
^{o}C	Degrees centigrade
OD	Optical density
p	Plasmid
PCR	Polymerase chain reaction
pg	Picogram
pro	Promoter
RenLUC	*Renilla* luciferase protein
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcriptase-PCR
SEM	Standard error of the mean
T	Thymine
T1	First generation of transformed *Arabidopsis* plants
TAE	tris-acetate EDTA
T-DNA	Transfer DNA

TIO	Two-in-one
Tris	tris(hydroxymethyl)aminomethane
Triton X-100	t-Octylphenoxypolyethoxyethanol
U	Enzyme units
mg	Microgram
ml	microlitre
mM	micromolar
UTR	untranslated region
V	Volts
v/v	Volume per volume
w/v	Weight per volume
WT	Wild type

Chapter 1

INTRODUCTION

1. Introduction

1.1. Flowering plants: A life-cycle

As the most prolific plant group, flowering plants form the largest group of terrestrial vegetation. Their life-cycle changes between a haploid gametophytic and sporophytic generation, the latter of which is dominant. The gametophyte generation comprises a three-celled male gametophyte (pollen) and a seven celled female gametophyte (embryosac), both morphologically distinguishable from the diploid sporophyte (Maheshwari, 1950). The sporophyte forms the majority of the plant body, including the shoots, roots and flowering stems, while the gametophytic phase of the life-cycle commences with the creation of the haploid megaspore and microspore by meiotic division of the diploid megasporocyte and microsporocyte (or mother cells),. The haploid spores produced from meiosis must go through additional mitoses in order to create haploid gametophytes, which produce male or female gametes. The combination of female and male gametes during double-fertilisation subsequently results in two differentiated products of fertilisation, the embryo and endosperm (Berger, 2008). The flowering plants life-cycle phases, haploid (gametophyte) and diploid (sporophyte), contribute to their evolutionary success and enable the development of novel characteristics through sexual reproduction (Walbot & Evans, 2003; Berger & Twell, 2011; Twell, 2011). As a bryophyte, mosses have the ability to regenerate their tissues. In these species every single moss gametophyte is developmentally totipotent. The two moss generations are physically connected, because the sporophyte grows on top of the gametophyte. The life cycle of moss consists of a dominant, haploid gametophyte stage and a small, diploid stage, which is partly dependent on the gametophyte for its nutrition. Gametophytes produce male and female reproductive organs antheridia and archegonia respectively. Male gametes or spermatozoids are motile, have flagella and produced within antheridia, whereas female gametes (oocytes or egg cells) are produced in archegonia. Fertilization is achieved by swimming of spermatozoids through a surface water film and down to the neck of the archegonia, which normally contains one egg cell. Gametes are produced via mitosis and fuse to form zygote. The zygote develops into a gametophyte-dependent sporophyte, which produces spores by meiosis

that produces further gametophytes (Cove *et al.*, 1997) (Figure 1.1).

1.1.1. Sexual reproduction of flowering plants

All sexually reproducing eukaryotic organisms utilise the process of meiosis during a particular phase of their life cycle. Sexual reproduction in flowering plants involves the transition from the sporophytic to the gametophytic stages through the process of meiosis (Bhatt *et al.*, 2001). Flowering plants go through various transitional phases throughout their life cycle, and the transition to the floral stage is one of the most notable, as the adult changes from vegetative to reproductive development. Angiosperm development is dissimilar to life in the animal kingdom, in which the germline is founded during early stages of embryogenesis and continues as a distinct stem-cell population (Strome & Lehmann, 2007; Hayashi & Surani, 2009). Indeed, angiosperms contain an undifferentiated population of stem cells as part of the apical meristems in the roots. These root alter between the creation of vegetative elements to reproductive structures, as well as signals that are triggered by environmental factors like the chemical and nutritional environment, daylength, the intensity and luminosity of light and temperature, in addition to endogenous signals that are transmitted by hormones (Parcy, 2005; Bäurle & Dean, 2006). Despite the fact that both sexes produce gametes that are comparable in the higher plants, the fate of each of the sexes is distinguishable. During the production of the male gametes all four products of meiosis, the microspores will develop to produce mature pollen grains (McCormick, 2004; Scott *et al.*, 2004). Conversely, during the production of the female gamete one of the four megaspores (the surviving megaspore) creates the usually seven-celled female gametophyte (embryo sac). Within the earliest stage of ovule development, a solitary megaspore mother cell (MMC) from the internal tissue, referred to as the nucleus develops. The MMC goes through a process of meiosis in order to generate four haploid megaspores, three of which undergo programmed cell death. Remaining proximal megaspores found at the chalazal section complete three stages of mitosis in order to create eight nuclei. Subsequently cellularization occurs to form seven cells, two of which are synergids, three of which are antipodal cells and one of which is an egg cell and another the central cell (Drews & Goldberg, 1989; Drews & Yadegari, 2002; Sundaresan & Alandete-Saez, 2010). For the male gametophyte, when the haploid

microspore divides in an asymmetrical manner to create a small germ cell (generative cell) that is contained within a larger vegetative cell, thus establishing the germline .This generative cell thenundergoes a last stage of mitosis to create two sperm cells. These cells are then transported through the pollen tube to the embryo sacs where they undergo double fertilisation (Weterings & Russell, 2004).

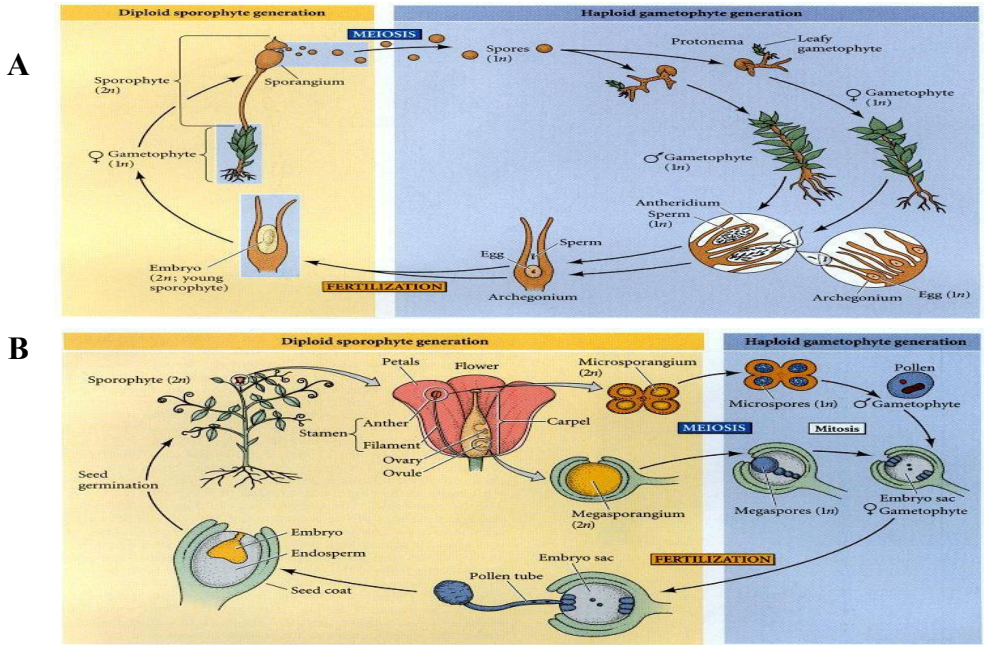


Figure 1.1: The life cycle of a land and flowering plants. Flowering plants have two separate stages of life cycle the diploid sporophyte creates haploid spores through the process of meiosis that later become the gametophyte generation. The microspores (male) go through a process of two distinct stages of mitosis development (PMI and PMII), which creates mature pollen that contains two sperm cells. The female megaspores go through three stages of mitosis in order to create seven-celled embryo sacs containing the egg cell (EC) as well as the central cell (CC). To achieve double fertilisation both of the sperm cells are transported to the embryo sacs through the pollen tube. One sperm fuses with the egg producing the zygote, and the second sperm fuses to the diploid central cell to generate the triploid endosperm which is needed to support the development of the embryo. The life cycle of moss consists of a dominant, haploid gametophyte stage (A) and a small, diploid stage, which is partly dependent on the gametophyte for its nutrition. Gametophytes produce male and female reproductive organs antheridia and archegonia respectively. Male gametes or spermatozoids are motile, have flagella and produced within antheridia, whereas egg cells are produced in archegonia. Fertilization is achieved by swimming of spermatozoids through a surface water film and down to the neck of the archegonia, which normally contains one egg cell. Gametes are produced via mitosis and fuse to form zygote. The zygote develops into a gametophyte-dependent sporophyte, which produces spores by meiosis that produces further gametophytes (Cove *et al.*, 1997). Adapted from (Gilbert ,2000).

1.1.2. Development of the male gametophyte

1.1.2.1. Microsporogenesis

During the process of microsporogenesis, the central L2 cell layer specifies an additional primordial cell, the archesporial cell that divides to create PPC (primary parietal cells) as well as PSC (primary sporogenous cells). Additionally, mitotic separations within the primary parietal cells create a number of concentric layers that make a distinction between the endothecium, the central layer and the most central tapetum. Surrounding the sporogenous cells, the tapetum feeds and provides nutrients for the nascent microspores throughout the process of pollen mother-cell meiosis as well as microspore maturity. Conversely, the primary sporogenous cells create the microsporocytes or the male meiocytes or alternatively the PMC. These PMC are then surrounded and subsumed by a semi-permeable wall of β-1, 3-glucan (callose), which separates the meiocyte cells from other sporophytic anther cells. After this the pollen mother cells go through a meiotic division in order to create a tetrad of the haploid microspores. These unicellular microspores are then released from the tetrad through the actions of a callose enzyme with endoglucanase and exoglucanase activities, which is located initially within the nutritive layer found within the stamen (tapetum) (Scott *et al.*,2004). Disruption of these enzymatic activities produce the *qrt* (*quartet*) mutant phenotype of which four microspores remain attached while the grains of pollen are shed in the form of tetrads. Overall, three of the genes are needed for the dissemination of tetrads, and these have been studied in *Arabidopsis* (*qrt1; qrt2; qrt3*). The identity of the molecules and the purpose of the *QRT2* gene is, as of yet, undetermined, however, the *QRT1* gene encodes a pectin methylesterase, while the *QRT3* encodes a polygalacturonase which is tapetum-specific that is involved in the collapse of the mother-cell wall, according to Preuss *et al.* (1994), Rhee *et al.* (2003) and Francis *et al.* (2006).

1.1.2.2. Microgametogensis

In new microspores, in which their nucleus is situated at the central point of the cell and various vacuoles are formed within the cytoplasm. Throughout microgametogenesis,

microspores grow and the smaller vacuoles converge to create a single, larger one; this is connected to the process of migration of the cell nucleus from the microspore to the periphery of the cell by the cell wall. (Owen & Makaroff, 1995; Yamamoto *et al.*, 2003). These microspores are polarised, and go through two stages of mitotic division. PMI (pollen mitosis I) is asymmetric two unequal cells the larger vegetative cell and the smaller generative cell. The vegetative has dispersed nuclear chromatin, accumulating a particularly dense cytoplasm (McCormick, 2004) that has within its structure proteins, lipids and carbohydrates. The small generative cell includes a greatly condensed chromatin, and also has within it few organelles as well as a store of metabolites for future use. The asymmetric division of the microspore is understood to be a vital element of germ cell fate specifications and vegetative cell fate is considered a default pathway for development (Eady *et al.*, 1995; Twell *et al.*, 1998). Both vegetative and generative cells have distinguishable nuclei and cytoplasma as well as an expression profile that is unique, which confers upon the cells their particular structure and fate. Before further mitotic division, the germ cell moves to the centre of the pollen and goes through an additional morphogenesis to gain a longer or else a 'spindle-like' shape, which considered to be upheld through the cortical structure of bundled microtubules (Palevitz & Cresti, 1989; Cai & Cresti, 2006). The cell in its vegetative state is arrested at the stage of G1 of the cell's cycle, though the smaller generative cell goes through an additional mitotic division, which is referred to as pollen mitosis II (PMII) in order to create twin sperm cells. A physical connection is established between the generative cell or the pair of sperm cells and the vegetative cell nucleus referred to as the MGU (the male germ unit), in bicellular and tricellular species (Dumas *et al.*, 1998; Lalanne & Twell, 2002). During maturation of *Arabidopsis* pollen, the vegetative cell accumulates lipid and carbohydrate reserves in addition to the transcripts and proteins, which are needed for the rapid pollen growth (Pacini, 1996).

Furthermore, the synthesis of disaccharides (sugars), amino acids (proline) and glycine betaine are used as an osmoprotectants for protection of cell membranes, as well as proteins that were used throughout the dehydration of the pollen (Schwacke *et al.*, 1999). The vegetative cell, nurtures the germ cell, and then later creates a pollen tube which delivers the sperm cells to the embryosac.

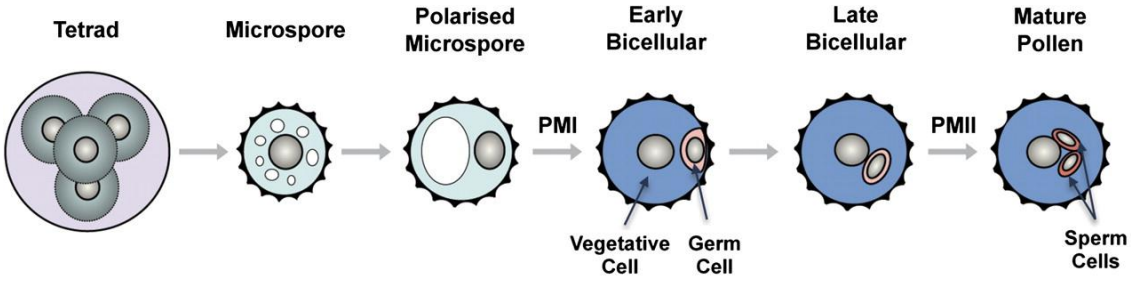


Figure 1.2: Pollen development in flowering plants. Schematic diagram that shown the morphological changes that take place within male gametophyte development in line with the timeline of the cell-cycle stages. Throughout microsporogenesis, diploid pollen mother cells go through meiotic division in order to create a tetrad of haploid microspores. These spores then go through a highly asymmetric mitotic separation referred to as Pollen Mitosis I (PMI), and this creates the large vegetative cell in addition to the smaller generative (germ) cell. The germ cell goes through an additional mitotic division referred to as pollen mitosis II (PMII) to create a pair of sperm cells. These cells then continue S-phase before attaining G2 stage by their arrival at the embryonic sac. Diagram adapted from Borg & Twell (2010).

1.1.3. Germ cell division

In plant species which shed bicellular pollen, the division in the generative cell (PMII) occurs within the pollen tube. Conversely, in species which shed tricellular pollen, the generative cell division occurs before anthesis. When PMII occurs before pollen I shed at anthesis, this involves involves a standard mitosis including the formation of the cell plate. In contrast, the division of the germ cell in the pollen tube, can result in spatial limitations and the cell plate may be tangential or longitudinally-orientated with regard to the pollen tube long axis (Terasaka & Niitsu, 1989).

The most explored element of germ cell division is the formation of the mitotic spindle within the metaphase plate and the mode of the cytokinesis. Within the pollen tubes from the monocot *Endymion non-scriptus* distribution of the microtubules as part of the generative cell that has been assessed. Furthermore, it was shown that, with the prophase phase, the cytoplasmic microtubules deteriorate and are replaced by the mitotic spindle fibres (Burgess, 1970). In the case of tobacco, an extremely lengthened cell profile as well as a condensed nucleated chromatin forms the pre-mitotic generative cells. This chromatin goes through an additional condensation at the time in which the cell goes through prophase. The microtubules are programmed to disappear from the cytoplasm within the generative cell of tobacco plant and *Ornithogalum virens* during

the period of time in which the mitotic spindle is present (Yu & Russell, 1993; Charzynska & Cresti, 1993). The angled orientation of the PMII mitotic spindle results in the atypical placement of chromosomes of the mitotic spindle (Palevitz & Cresti, 1989; Taylor *et al.*, 1989; Terasaka & Niitsu, 1989; Palevitz, 1990; Yu & Russell, 1993). Within species such as *Tradescantia* and *Rhododendron*, chromosomes are distributed in a perpendicular fashion with regard to the angled metaphase spindle (Palevitz & Cresti, 1989; Taylor *et al.*, 1989; Palevitz, 1990; Liu & Palevitz, 1991) at the same time in *Hyacinthus*, there is regular distribution of kinetochores across the equatorial plate (Del Casino *et al.*, 1992). In the case of tobacco, the kinetochore fibres are allocated in an irregular fashion across the side and the depth of the germ cell (Yu & Russell, 1993). The meta-phase spindle of the species *Ornithogalum virens* is generally comprised of kinetochore fibres; these are found in a single place, which is found to be perpendicular to the polarised point of the spindle axis (Banaś *et al.*, 1996). In the case of *Ornithogalum virens*, anaphase is denoted by a reduction in the length of the kinetochore fibres, as well as a significant lengthening of the mitotic spindle, this is the notable feature of the germ cells throughout the bicellular as well as the tricellular species (Yu & Russell, 1993; Charzynska & Lewandowska, 1990; Banaś *et al.*, 1996).

The process of cell division at PMII is described in both tri-and bicellular pollen of a number of plant species which include; *Nicotiana tabacum* (Palevitz, 1993; Yu & Russell, 1993); *Rhododendron* (Taylor *et al.*, 1989); *Hordeum vulgare* (Charzyńska *et al.*, 1988); *Ornithogalum* (Banaś *et al.*, 1996); *Lilium* (Xu *et al.*, 1990) and *Brassica napus* (Charzynska *et al.*, 1989). As is the case in these plant species, cytokinesis at PMII also includes the formation of a phragmoplast and cell plate prior to dividing the cytoplasm. Despite this, cytokinesis in the case of *Hyacinthus* and *Tradescantia* are not thought to generate a cell plate or phragmoplast. In *Tradescantia*, the process of cytokinesis seems to involve the process of furrowing, as found by Del Casino *et al.*, (1992) as well as by Palevitz & Cresti (1989). Among the most notable elements of the cytokinesis of the generative cells is that there is no presence of F-actin (Palevitz & Tiezzi, 1992), which suggests that the actin filaments do not need to function in cell plate creation with regard to PMII.

1.2. Analysing male gametophyte gene functions

1.2.1. Screening process of the male gametophyte mutants

The determination of functions of the genes at different stages of pollen development is an important topic of research for *Arabidopsis*. Assessment of the genetics of the mutant gametophyte is still somewhat of a challenge as not every one of them is scorable by simple observation, with regard to the mutant phenotype. Due to the fact that the particular lethal male gametophytic mutants are transmissible only through the female to generate heterozygotes, plants homozygous for the mutation are unrecoverable. Additionally, the phenotype of the plants that carry heterozygous mutations commonly appear phenotypically normal and thus demand additional assessment for the purposes of identification of the mutation (Procissi *et al.*, 2001). In a number of studies screens have been devised for the purposes of detecting mutations with effects on gametophytic viability, often resulting in a a low percentage of entirely defective male mutated gametophyte cells (Feldmann *et al.*, 1997; Bonhomme *et al.*, 1998; Howden *et al.*, 1998).

Regardless of this fact, many forward and reverse (from the phenotype-to-gene and gene-to-phenotype respectively) approach have been found to be promising with regard to the isolation of gametophytic mutations and genes that are influential in the development of the functions of the male gametophyte. A couple of forward-based means of dealing with this have made use of direct detection of abnormal cells by morphological screening of the pollen with the employment of histo-chemical staining with regard to the DNA (Chen & McCormick, 1996; Park *et al.*, 1998) and callose (Johnson & McCormick, 2001). The alternative means of dealing with this is through the exploitation of a segregation ratio distortion to identify those mutations that have undergone a reduction in the transmission through either male or female gametes (Bonhomme *et al.*, 1998; Howden *et al.*, 1998; Grini *et al.*, 1999; Procissi *et al.*, 2001; Johnson *et al.*, 2004; Lalanne *et al.*, 2004).

1.2.1.1. Analysis of segregation distortion

The reprocess of insertional mutagenesis generally utilises DNA elements which can

insert themselves randomly within the structure of the genome. For example transposons or the T-DNA which include dominant antibiotic or herbicide resistance markers. The screen that is employed for segregation distortion is founded on the proof that if an insertion disrupts an essential female or male gametophytic gene sequence, then the subsequent reaction of resistance with regard to sensitivity of an individual (self) progeny will alter between the lower-than-expected reaction of segregation at 3:1. In the situation of the hemizygous T-DNA/transposon line that separated at a 1:1 ratio for the purpose of resistant progeny to sensitivity, then this would suggest that the insertion did not manage to transmit through the male or the female gametophyte. As a result, the ratio for the distortion of the separation may be employed as a rough indication of the defect in the gametophyte genes (Howden *et al.*, 1998). Screening for the segregation ratio alteration function has resulted in the identification of several gametophytic mutated phenotypes that are the result of the T-DNA insertions by Feldmann *et al.* (1997), Bonhomme *et al.* (1998), Howden *et al.* (1998), Grini *et al.* (1999) and Johnson *et al.* (2004) or else the transposons; (Lalanne *et al.* 2004 and Oh *et al.* 2003).

A collection of unique transformants from *Arabidopsis* was created using seed co-cultivation (Feldmann, 1991; Feldmann *et al.*, 1997). The first screening of the 142 samples of transformed lines on the media than contained the selective antibiotic (kanamycin) ended in the identification of seventeen lines which did not show Mendelain segregation for the dominant Kan^R trait (Feldmann *et al.*, 1997). Screening of T-DNA lines by Howden *et al.*, (1998) resulted in the selection of eight separate T-DNA lines with putative gametophytic mutations that are reproducibly showed to be distorted with the segregation ratio 1:1. Inclusive within these are the cellular morphogenesis mutation *limpet pollen* (*lip*) mutation which does not complete germ cell migration and a singular progamic stage mutant along with four lines that showed notable effects on male and female transmission (Howden *et al.*, 1998). Genetic analysis of thirty-eight T-DNA Tdt lines by (Bonhomme *et al.*, 1998) proved that eight were male-specific mutations with the T-DNA transmission throughout the pollen at 0-1%. A different methodological approach was founded on the segregated distortion of the almost-visible marker of the screen in the case of EMS-induced mutated gametophytes. An *Arabidopsis* line that is multiply marked on, chromosome I (mm1)

carried with it five visible recessive markers all of which were employed in the identification of gametophytic mutations that existed close to the markers. Seven independent mutants showed a significant transmission reduction through the male *mad1*(male gametophytic defective), *mad2, mad3* and *mad4*) or else through male and female *bod1*(both male and female gametophytic defective), *bod2,* and *bod3),* which show pleiotropic phenotypes (Grini *et al.,* 1999).

In the case of an alternative screening method, gametophytic mutations were shown to be identifiable by segregation ratio distortion with the use of a herbicide resistance marker (Basta) and a T-DNA, that contains the LAT52:GUS reporter gene that is pollen-specific (Twell *et al.*, 1990; Twell *et al.*, 1989), and which allows for an autonomous tag for pollen grains that contain an insertion. The screen was performed within the genetic background of *quartet* and led to the identification of 32 haploid-disrupting heterozygous mutants that were referred to as *hapless* (*hap*), and defined specific genes for the development of the pollen grains, as well as pollen tube growth and guidance in the pistil and ovary (Johnson *et al.*, 2004). In another method, the insertion of transposons was used and the Ds transposon insertion lines with solitary enhancer trap (DsE) or gene-trap (DsG) elements harbouring kanamycin resistance were screened for segregation ratio distortion (Sundaresan *et al.*, 1995; Moore *et al.*, 1997). This approach was used to try and identifying the transposon-tagged genes that possessed gametophytic roles (Moore *et al.*, 1997; Page & Grossniklaus, 2002), especially with the intention of identifying genes that are needed for post-pollination development of pollen in the Arabidopsis. From the sample population of 3359 Ds transposon lines of insertion, there were twenty separate lines that demonstrated a stable yet-reduced segregation ratio. Included in these were ten of the *ungud* mutations, which affected both female and the male gametogenesis and nine *seth* mutations, which had an effect on pollen functions during the progamic phase, with one male-specific mutation dubbed *halfman* which was seen to influence microsporogenesis (Lalanne *et al.*, 2004; Oh *et al.*, 2003).

1.2.1.2. Screening of pollen cytology

Both chemically-induced or radiation-induced mutagenesis have been used to generate

mutants in pollen development in *Arabidopsis*. Chemical mutagens such as ethyl methanesulfonate (EMS) have the advantage of generating single nucleotide changes while small deletions can be generated with the use of UV or X-ray radiation. EMS is used generally in the creation of G/C and A/T point mutations, and these can lead to an entire loss of generic function through the early stop-codon, or alternatively be used as a critical amino acid.

Indeed, physical and chemical mutagenesis (Chen & McCormick, 1996; Park *et al.*, 1998; Lalanne & Twell, 2002; Johnson & McCormick, 2001) have both been employed for the purpose of identifying lesions within the genes that are part of the development of the male gametophyte. When the pollen taken from the mutated lines of high fast neutrons and EMS, is morphologically screened, they yield a broad range of notable phenotypes that are affected at different stages of the development of pollen. A morphological screening process has found on the *Arabidopsis* No-0 (Nossen) background seeds, which are subjected to fast neutrons, which identify the *sidecar pollen (scp)* mutation that influences the microspore asymmetry and the fate of the cell. Pollen that was isolated from the M1 plants was stained with the use of DAPI (4‘, 6-diamidino-2 phenylindole), and this scored as positive with regard to pollen phenotype. Heterozygous *scp* mutated plants were noted on the basis of them having unnatural pollen morphology in the case if almost half of the sample population (Chen & McCormick, 1996). In a similar, in the case of the EMS-based pollen phenotypically screening, this resulted in the isolation of the pollen mutations in which the grains of the pollen were also stained for callose prior to anther. The mutant *raring-to-go* (*rtg*) gametophyte was noted as a rare mutation in which some pollen was seen prematurely hydrated germinates, and taken from the pollen tubes of the anther. In alternative pollen screen that was taken form an EMS-derived, the pollen grains were analysed from many mutations with deviate symptoms from the tri-cellular pollen morphological set. A matured pollen sample was taken from a set of 10,000 mutagenized M2 plants. (Nossen, No), and these were also stained with the use of DAPI, before being assessed for any abnormal pollen cell separation phenotypes with the use of fluorescence microscopy. This genetic screen managed to examine fifteen to twenty separately induced mutations, which influenced the standard pollen division as well as the intracellular structure of the pollen (Park *et al.*, 1998). Mutations that were noted from this screening process were

set into groups on the basis of these three criteria; 1) mutations that affected microspore division cytokinesis and symmetry; 2) mutations that influence the male germ unit and 3), mutations that influenced germ cell division. Asymmetry division affected by the *gemini pollen1 (gem1)* that produced twin sperm cells at PMI. Within the MAP215 family, the MOR1/GEM1 was a member if the microtubule-associated proteins, those which are involved in the microtubule assemblage, and which are related to the inter spindle and phragmoplast array of microtubules (Park *et al.*, 1998; Park & Twell, 2001; Twell *et al.*, 2002).

The *two-in-one pollen (tio*) mutation that was formerly named *solo pollen*, did not manage to go through ordinary cytokinesis at PMI. TIO is the plant homologue of the Ser/Thr protein kinase FUSED, and is localised to the middle-line of the phragmoplast, and here it plays a vital role in the expansion of the cell plate (Oh *et al.*, 2005). Morphological screens identified a distinct set of pollen mutations that demonstrated defects, in positioning of the male germ unit (MGU). This was called the *germ unit malformed* and *male germ unit displaced* (*gum* and *mud* respectively). In the case of *gum* mutations, the vegetative nucleus is situated in an adjacent to the pollen grain wall, which is distinct from the two sperm cells. However, in the case if the *mud* mutant, the MGU is positioned at the wall of the pollen grain (Lalanne & Twell, 2002). Analysis of the genetics showed that each of these mutations demonstrated that there was a lower pollen transmission, therefore, these act as gametophytic mutations. Additional cytological assessment showed that the correct MGU assemblage is needed of the efficient transmission of male gametes through the pollen grains (Lalanne & Twell, 2002).

The most significant mutations were identified by the use of the EMS mutagenesis screening process, which generated a bicellular pollen phenotype at anthesis thus was termed *duo pollen (duo*) This *duo* class was comprised of *duo1, duo2, duo3, duo4, duo5* and *duo6,* all of which arose in different M_1 parental groupings (Durbarry *et al.*, 2005). *duo* mutations shed bicellular pollen grains, which contained a vegetative nucleus as well as an undivided germ cell. These *duo* mutations were identified with the use of the screen and subsequently were separated as heterozygous mutations, before being mapped to the alternative chromosomal locations. The initial grouping consisted of the *duo 1-3* mutants with a rounded germ nucleus at the stage of anthesis. The heterozygous

duo 1 and *duo 2* mutants produce roughly 50% bicellular pollen. The phenotypic observation indicated that the *duo2* germ cell entered mitosis and arrested at prometaphase, conversely, the *duo 1* cells completed their S-phase, though did not manage to enter mitosis (Durbarry *et al.*, 2005). The second grouping included *duo 4-6* in which the mature pollen contained elongated single germ cell nuclei. The asymmetric division of the microspores was complete within these mutants, though the germ cell failed to enter division at PMII (Durbarry *et al.*, 2005).

1.2.1.3. Taxonomic and phylogenetic significance of pollen cell number

The formation and fitness of two sperm cells during male gametophyte development in flowering plants is critical for double fertilization. The timing of the formation of the two sperm cells is different in different plant species (Friedman, 1999). In approximately 70% of angiosperms PMII takes place after anther dehiscence resulting in release of bicellular pollen whereas in more advanced angiosperm families pollen is shed in a tricellular condition. Schurhoff first realized the phylogenetic implications of the distribution of both bicellular and tricellular pollen. His idea that the tricellular pollen condition could be phylogenetically evolved from bicellular ancestors was practically exercised by two embryologists (Schnarf & Wunderlich, 1939) who published a summary of all available data on the taxonomic distribution of binucleate and trinucleate pollen in angiosperms. Later (Brewbaker, 1967) conducted the first extensive survey of about 2000 angiosperm species and confirmed Schurhoff's hypothesis that tricellularity has evolved from the ancestral bicellular condition. The phylogenetic significance of pollen nuclear number as suggested by Schurhoff and later confirmed by Brewbaker resulted in the Schurhoff – Brewbaker Law Webster & Rupert,(1973), which states that (1) bicellular pollen is primitive in the angiosperms as a whole and within individual taxa; (2) trinucleate pollen is derived independently from bicellular pollen in all instances; and (3) the shift from trinucleate to bicellular is irreversible. Webster *et al.*, (1982) validated the Schurhoff- Brewbaker Law, in Euphorbiaceae tribe Euphorbieae presence of both bicellular and tricellular pollen was strongly associated with taxonomic groupings and tricellular pollen was independently derived from plesiomorphic bicellular pollen. Similar observations were reported by

Grayum, (1986) and according to his studies the distribution of bicellular and tricellular pollen in the monocot family Araceae is in complete accord with the Schurhoff-Brewbaker Law although Grayum, (1986) also found low numbers of tricellular pollen.

1.2.1.4. Heterochrony and pollen cell number

The variability in the timings of cell cycle events in different angiosperm species may be attributed to heterochronic shift in the germ cell cycle events throughout the evolution of pollen development (Friedman, 1999). Different patterns of sperm cell cycle can be found in bicellular and tricellular species. A portion of angiosperm species shed bicellular pollen in the interphase of the germ cell cycle and PMII occurs entirely after anthesis in the pollen tube. The gametic fusion takes place in G1 of the sperm cycle resulting in 2C zygote which then goes through S phase prior to first zygotic division (Friedman, 1999). This pattern is followed by species of the ANITA grade including *Nuphar*, *Illicium* and *Kadsura* (Williams & Friedman, 2004; Friedman *et al.*, 2002). A variation to this ancestral cell cycle has been found in the advanced bicellular family Solanaceae in which bicellular pollen is shed in a reportedly arrested mitotic state probably at prophase of the cell cycle. The gametic fusion occurs in G2 phase with sperm cells in 2C DNA state resulting in 4C zygote ready for the first zygotic division (Tian *et al.*, 2005). In the remaining 30% tricellular plant species, the male germ cell cycle has diverged into three different patterns, in which PMII occurs within the pollen grain and pollen is shed with sperm cells in G1, S or G2 phase (Friedman, 1999). The heterochronic shift in the male germ cell cycle progression in favour of tricellular pollen may have been a result of specific shortening of male germ cell cycle involving change in certain germ line regulators. The change in male germ cell cycle in favour of tricellular pollen may be a result of consistent environmental factors such as temperature, speeding up the cell cycle machinery and resulting in a polymorphic pollen state, a condition reported in some extant angiosperm species (Lora *et al.*, 2009). Certain physiological differences exist between bicellular and tricellular pollen. Pollen bicellularity is quite regularly associated with gametophytic self-incompatibility (Brewbaker, 1957), long-term pollen viability (Brewbaker & Majumder, 1961), and the presence of a wet stigma (Heslop-Harrison & Shivanna, 1977). In contrast tricellular pollen in general shows rapid germination rates, shorter longevity (Brewbaker, 1967),

and often sporophytic self-incompatability systems with dry stigmas have been found in tricellular species. Furthermore, like pollen bicellularity, gametophytic self-incompatibility has been regarded as the pleisomorphic state and corresponds to the widespread occurrence of bicellularity in pollen (Heslop-Harrison, 1983).

Various factors that contribute to the formation of bicellular and tricellular pollen at anther dehiscence may include temperature, humidity as well pollen physiology and pollen developmental stage. Considering these factors, if pollen stays longer in a hydrated state, in hot and humid surroundings, it may lead to the formation of tricellular pollen at anther dehiscence, which if it persists could lead to the fixation of the trait of pollen tricellularity (Lora *et al.*, 2009).

1.3. Coexistence of polymorphic pollen types in angiosperms

The presence of both bicelllular and tricellular pollen at anther dehiscence was reported by Maheshwari in some unrelated species in 1950. Brewbaker (1967) reported all basal angiosperm families as exclusively bicellular but later the presence of tricellular pollen was reported in some extant basal angiosperm species i.e. *Annona cherimola* from family Annonaceae (Rosell *et al.*, 1999) as well as in the fairly primitive order Alismatales (Grayum, 1986), Laurales (Gardner, 1975) and the dicot family Euphorbiaceae (Webster & Rupert, 1973). The idea of environmental manipulation of the pollen nuclear number was previously refuted and referred to as "genetic variability" by Grayum (1986) but recent studies have shown that environmental conditions favouring high humidity and adequate temperature for germ cell division favours the production of tricellular pollen at anthesis in *A. cherimola* (Lora *et al.*, 2009). Further research on gene expression during pollen mitosis II and pollen dehydration may provide answers about the evolutionary shift from bicellular to tricellular pollen.

1.4. Classification of the family of Magnoliaceae

Experts in taxonomy have long questioned the classification of lineages within Magnoliaceae, with the exception of *Liriodendron*, a very distinct genus. The second subfamily of this group is referred to as Magnolioideae, though current research into advanced DNA sequencing (Qiu *et al.*, 1995a; Qiu *et al.*, 1995b; Azuma *et al.*, 1999; Azuma *et al.*, 2001; Kim *et al.*, 2001; Nie *et al.*, 2008) along with in-depth morphological investigations has discovered that this subfamily contains only one known genus – *Magnolia* (Figlar, 2002a; Figlar, 2002b; Sima, 2001; Baranova & Jeffrey, 2000; Nooteboom, 1985; Nooteboom, 1993; Nooteboom & Chalermglin, 2000; Li & Conran, 2003; Figlar & Nooteboom, 2004). James E. Dandy, renowned taxonomist based at the British Museum of Natural History, conducted extensive research into the Magnolioideae subfamily and his system comprising 11 genera was highly regarded by taxonomists during the 20th century. This system was further developed in 1927 as the subfamily was said to comprise four major genera, namely *Magnolia*, *Manglietia*, *Michelia* and *Talauma,* along with several more minor genera, namely *Aromadendron, Kmeria*, *Pachylarnax*, *Alcimandra,* and *Elmerrillia.* At a later stage, two additional genera were included *Paramichelia* and *Tsoongiodendron.* This system was devised based on the research observations made by Dandy along with reference to the work of other taxonomists in the field. This classification states that plants with at least two ovules or seeds on each fruiting carpel form part of the *Manglietia* genus. Those with flowers located near axils and stipitate gynoecium form part of the *Michelia* genus and those whose carpels disintegrate when releasing seeds, as opposed to dividing in half form, part of the *Talauma* genus, a genus comprised of woody fruit plants. For *Magnolia*, Dandy distinguished this genus by plants with terminal flowers and one or two ovules for each carpel, which separate lengthwise upon the release of seeds. The minor genera represented variations of the primary genera. For instance, *Elmerrillia* has the same characteristics as *Michelia* except it has a sessile gynoecium while *Paramichelia* is distinguished from *Michelia* only by its fruit. Dandy's system also differentiates between the two species, which display unisexual flowers, and this genus is known as *Kmeria*. This system was devised in 1974 and was well received by taxonomists who accepted Dandy's classification containing 10 genera, the most significant of which was *Magnolia*. However, Liu Yu-Hu who is a

Chinese expert in Magnoliaceae made slight adjustments to the system in 1984. Four additional genera were incorporated into Dandy's system, as delineated in *Magnolias of China* (Liu & Garden, 2005). Nooteboom, (1985) believed that Dandy made a number of errors in his observations, perhaps on account of homoplasies, particularly in relation to the attributes of fruit. Thus, he proposed that *Talauma* be included, as part of *Magnolia* and that two of the minor genera should be classified as *Michelia*. Based on Nooteboom's system, Magnolioideae contained six different genera (*Magnolia, Manglietia, Michelia, Elmerrillia, Kmeria,* and *Pachylarnax*) and this system was accepted by most taxonomists with the exception of the Chinese who still adhered to the classifications made by Liu (2005).

DNA sequencing provided a means to more accurately determine plant characteristics in the late 20th century as comparative analysis of DNA sequence alignment allowed taxonomists to create relationship trees, more formally known as molecular phylogenies, which reflect and measure the extent of relationships that exist between different families or genera. According to Judd *et al.* (2008), this method has become popular amongst taxonomists as the majority agree that classifications must identify groups, which contain ancestral species and all its descendants, monophyletic groups. Analysis of these molecular phylogenies in terms of Magnolioideae indicates that *Manglietia, Michelia* and the three minor genera are closely interlinked with sections of *Magnolia*. In fact, *Michelia* is found to occupy a clade that also contains *Magnolia,* a section of *Magnolia* (Kim *et al.*, 2001; Azuma *et al.*, 2001). Furthermore, these reconstructions indicate that *Michelia* resemble *Yulania,* a subgenus of *Magnolia* and *Manglietia* closely resembles *Oyama* and *Rhytidospermum,* two sections of *Magnolia*. Using this information in conjunction with morphological data, a further classification system for Magnolioideae was proposed by Figlar & Nooteboom (2004), which contained *Magnolia* as the only genus. This system incorporated the 13 constituent clades into sections and subgroups of the genus. Nonetheless, it was also posited that 13 genera could have been proposed using this approach, as rank has no impact on monophyly; however, this suggestion was discounted due to low inter-clade sequence divergence (0.63%) as many different clades appeared practically identical. In addition, this suggestion was rejected, as a monogeneric system would not require extensive nomenclature changes. In effect, using a single genus, only *Manglietia, Michelia* and

the three minor genera would need to be renamed whereas the entire genus of *Magnolia* would need to be renamed along with the ten genera using the 13 genera system. This would inevitably impact upon the legacy of the *Magnolia* genus and the extensive amount of taxonomic research that has already been conducted on the topic (Figlar, 2009)

1.5. Gene expression studies in male gametophyte

Completion of the *Arabidopsis* genome sequence (the Genome Initiative 2000) as well as the relevant databases such as the TIGR *Arabidopsis thaliana* database and the Arabidopsis Information Resource (TAIR), made the availability of the many transcriptomic sets of data do the transcriptome of the pollen (Honys & Twell, 2004; Pina *et al.*, 2005), and the contemporaneously available information for the sperm cell transcriptome (Borges *et al.*, 2008). Availability of the pollen spesific toolsthat were needed for the research (Johnson-Brousseau & McCormick, 2004) which are significant advances that will assist in the deciphering of the role of the many important genes involved in the pollen development.

1.5.1. Studies of the pollen transcriptome

The first evidence that led with certainty to a discernible conclusion with regard to the gene expression in pollen development was founded on the substance isozyme (Pedersen *et al.*, 1987) and RNA hybridisation investigation (Mascarenhas, 1990), that specify that the extensive pollen gene expression programme. Emerging technologies that allow for higher-throughput such as microarrays (Hennig *et al.*, 2003; Honys & Twell, 2003), and a serial analysis of gene expression (SAGE) technology (Lee & Lee, 2003) as well as expressed sequence tags (EST) (Engel *et al.*, 2003) have allowed for a more comprehensive assessment of the male gametophytic gene expression. Within these methods, the microarray remains one of the most proficient and normatively utilised means of identifying the range of transcripts within the targeted issue. Within the pioneering study field, there are two distinct platforms what are used to explore the matured pollen transcriptome inclusive of the serial analysis of genetic expression

(SAGE) tech (Lee & Lee, 2003), as well as the GeneChip microarray (Honys & Twell, 2003; Becker *et al.*, 2003). This latter approach was used to exploit the 8K Affymetrix AG microarrays, and provided assessment for mature pollen from the *Arabidopsis* ecotype Landsberg *erecta* (Ler) on the basis of around 30% of the *Arabidopsis* genome (Honys & Twell, 2003). Expression data that was obtained from the 8K GeneChip array was indicated to the significant at 61% overlap of the pollen transcriptome with regard to the sporophyte. Furthermore, with additional improvements in the tech, the improved Affymetrix 23K *Arabidopsis* ATH1 array, which contained 22,591 genes permitted the analysis of in excess of 80% of the *Arabidopsis* genome (Redman *et al.*, 2004). Within an example study, the new ATH1 array was utilised in order to create the transcriptome information that covered four additional stages of male gametophyte development namely, bicellular pollen, tricellular pollen uninucleate microspore, and mature pollenfor Landsberg *erecta* (Honys & Twell, 2004). In additional two studies (Pina *et al.*, 2005) and (Zimmermann *et al.*, 2004), there were reported of the ATH1 data sets in the case of mature pollen as taken from the ecotype Columbia. The assimilated assessment of these sets of data in addition to the final normalised data identified from 5000-7000 genes that were expressed within the mature male gametophyte. This extended to the assessment to four stages of pollen development, with the entire number of genes that were expressed throughout the development of the pollen and their functions, which were predicted to be in excess of 14,000. Unexpectedly, 5% of the characterized genes demonstrated that they were strictly pollen-specific in their expressive patterns, as was found by Honys & Twell (2004) and Borg *et al.* (2009). An additional use of the findings was seen to be a slow decrease in the amount of the expressed genes from the earlier stages in which 12000 active genes were seen on microspore and bicellular pollen to the later stages in which a mere 7000 genes were seen to express in tricellular and mature pollen. These genes expressed at the late phase of pollen development are primarily included in the metabolism of the cell wall, the cytoskeleton and the cell signalling, which demonstrates the functions as specialised within the pollen in expectation of the maturity and the rapid pollen tube growth (Borg *et al.*, 2009; Honys & Twell, 2004). Within another important experiment, the microarray data sets were generated fromisolated sperm cells.

Pollen from the Columbia (Col-0) ecotype of *Arabidopsi*s that expressed the GFP

marker driven by the germline-specific *GEX2* promoter, which was mechanically disrupted and then subject to a fluorescence-activated cell sorting (FACS). The pollen that was disrupted to free the sperm cells before the GFP-marked sperm was purified, on the basis of the GFP signal, as well as their size and the existence of DNA. The data of the microarray sets of data were additionally generated for the seedlings as well as pollen as a repeat analysis. Overall, 5,829 genes (27% of the whole sample of 22,392 genes) within the ATH1 array gene transcripts were detected precisely within the sperm cells in comparison to the 33% and the 66% of the genes within the pollen and the seedlings, respectively speaking. By far the largest aspect of the genes (a number of 3,813) that were expressed within the sperm cells was additionally seen in the pollen cell on vegetation, in line with findings from (Borges *et al.,* 2008). Furthermore, the male gametophyte-specific genes are generally more identifiable through their high expression levels, which demonstrate the importance with regard to the specialised gametophytic programme. The transcriptomic data sets are available in abundance now, and so it is easy to investigate reverse genetic targets identified based upon founded on the compression with the overcoming of redundant genetic material the expression of individual genes within a family (Honys & Twell, 2004; Honys *et al.*, 2006).

1.5.2. Male germline gene expression of flowering plants

The revolutionary research with regard to the gene expression within the male germline mainly focussed on species that have larger and more easily obtainable and accessible germline, inclusive of the plants used in this study such as; maize, lily and *Plumbago* (Tanaka, 1988; Russell, 1991; Zhang *et al.,* 1998; Uchiumi *et al.,* 2006). The means of acquiring the relatively larger germline cells of both the tri- and bicellular plant species mentioned above allowed for the development of new techniques with regard to isolating and purifying these plants' male germline. In order to pout a germline in isolation, the pollen grains *in vitro* or else grown pollen tubes are initially subject to agitation of their enzymes, which is then followed by a separation of the germline cells from the rest of the cell remaining though a density gradient centrifuge (Tanaka, 1988; Xu *et al.,* 2002), as well as micro manipulative techniques (Zhang *et al.*, 1998; Chen *et al.,* 2006) and also fluorescence activated sorting of the cells (FACS) (Engel *et al.,* 2003).

Additionally, the cDNA libraries and the EST (expressed sequence tag) projects for sequencing are also taken from extracted germline cells, and this allowed for the first insight into the unique male germline transcriptome. Comparing the assessment of the male germline transcriptome data sets, which are generated from tobacco maize, lily and *Plumbago,* all of which indicated an overlap of expressed genes within the taxa, and the expression of the related classes of genes within their germline. Additional investigation which focuses on male germline identity, which ended in the investigation of the germline-specific transcript inclusive of the LGC1 *Lily Generative Cell1* (Xu *et al.*, 1999), as well as a polyubiquotin gene LG52 (Singh *et al.*, 2002) and finally the *GCS1* (Generative Cell Specific1) which were taken from the germ cells of the lily plant (Mori *et al.*, 2005). A number of the male germline-specific genes were seen to be homologous to those which have been expressed in the germlines of other species, and as can be characterised by *Arabidopsis*. Full homologues of the lily gene *GCS1* (*HAP2*), as well as three genes that were seen to be homologous to the maize sperm cells were expressed as the *GEX1*, *GEX2*, and *GEX3* genes that were also examples as those, which identfied in *Arabidopsis.* The *GCS1/HAP2* genes that were found to be encoded gamete surface protein were needed for the guidance of the pollen tube and the gamete fusion stage.It also represented a conserved male germline of a transcriptional signature within flowering plants (Engel *et al.*, 2005; von Besser *et al.*, 2006; Mori *et al.*, 2005; Alandete-Saez *et al.*, 2008).

1.6. Expression of transcription factor (TF) families during pollen development

On the whole, the prolific nature, and the variable nature of transcription factors is greater in plants than it is in any other kingdom. Roughly, 5% of the *Arabidopsis* genome codes (approximately 1533 genes) was encoded in putative transcription factors (Riechmann *et al.*, 2000). In the case of the germline of the *Arabidopsis* plant, the early and the late genetic expression programme needed a coordinated regulation within the level of transcription. In the case of the 1350 transcription factors within the *Arabidopsis* genome, this anchored on the Affymetrix ATH1 Genome Array, a total of 612 of the total permitted a reliable expression which, in the development of the male

gametophyte (542 in the early and 405 in the later stages) (Twell *et al.*, 2006).

In accordance with Honys and Twell (2004) the most over overrepresented transcription factor families within in the male gametophyte excess of twenty five members including members of the following families, CCAAT, C_2H_2 zinc finger, GRAS, bZIP, WRKY and TCP. Conversely, the MADS, IAA, HSF, HB, bHLH, NAC, AUX, AP2-EREBP, R2R3-MYB, and C_2C_2 zinc finger families were all underrepresented. Two main groups of TFs could be identified. One of the most important groupings among the early-stage expressed genes were seen to belong to the NAC, WRKY, ARF, TCP, Aux/IAA, HMG-box and Alfin-like transcription factor families. In the case of the second, a smaller grouping was composed of the C_2H_2 and the TUBBY transcriptional factor families, which mostly showed later profiles in expression (Honys and Twell, 2004). The members of the MYB-type of the transcription factor family demonstrated that higher expression in sperm cells (Borges *et al.* 2008). In addition to this sperm cell of a specified transcription factor that of At4g35700 was showed to be the single sperm cell in which the transcriptome was belonging to the C_2H_2 zinc finger family, which demonstrated the highest expression in all the transcriptional factors.

1.6.1. Transcription factors family within plants: the role of MYB domain

The v-MYB gene, which is seen in the avian myeloblast virus, was the first MYB that was identified (Klempnauer *et al.*, 1982). Assessment of the entire *Arabidopsis* genome sequentially has resulted in the identity of roughly 198 genes within the MYB, super-family a majority of which encode proteins with two repeats tryptophan-rich repeats. The plant proteins such as the MYB protein are referred to as such as the R2R3-MYB proteins. Plant genes of the MYB type encode the protein with DNA-binding domains, referred to as MYB domains. These domains are consisted of 1-3 conserved motifs, the residues of roughly 52 amino acids. Repeated motifs such as these and the MYB domains are bound to major grooves of the target DNA though the helix-turn-helix composition, and they interact with the bases and the phosphates.

Regularly spaced tryptophan residues can be seen, and these forms a tryptophan

conglomeration, within the helix-turn-helix, this characteristic is indicative of an MYB repeat (Saikumar *et al.*, 1990). With regard to the number of MYB repeats, MYB transcription factors may be subdivided into several sub-families; 1R, 2R (R2R3), 3R (R1R2R3) and 4R (R1R2R3R4) MYBs. Each of these contains 1,2,3 or 4 MYB repeats, respectively, with R2R3 MYBs as the biggest sub-family (Stracke *et al.*, 2001; Dubos *et al.*, 2010). A phylogenetic contrast of the rice and the *Arabidopsis* MYB family indicate fast expansion within the *Arabidopsis* MYB super-family following its separation from monocots. The *Arabidopsis* genome has overall, 198 MYB genes, from these 126 are R2R3-type, 5 are MYB3R, 64 are MYB-related with three being atypical; MYB genes (Yanhui *et al.*, 2006). The foremost of these MYB genes were seen in a *Zea may* and was called ZmMYBC1 (C1), and this is needed by anthocyanin synthesis within the aleurone of maize (Paz-Ares *et al.*, 1987). Those within the MYB family are included within several functions including phenylpropanoid metabolic processes (Grotewold *et al.*, 1994), biotic and abiotic stress (Segarra *et al.*, 2009), and the shape changes of the cell such as *Antirrhinum majuspetals* MIXTA (Noda *et al.*, 1994), cell differentiation (Oppenheimer *et al.*, 1991; Kang *et al.*, 2009), hormones response i.e. *AtMYB2* (Urao *et al.*, 1993), GAMYB and *CpMYB* (Gubler *et al.*, 1995), the creation of the of B-type cycling (Ito *et al.*, 2001) or else as part of the reactions of the plant i.e. *NtMYB1* (Yang & Klessig, 1996; Liu *et al.*, 2008). Many of the R2R3-MYB proteins have been seen to have an effect on plant development. *AtMYB33* and *AtMYB65* seem to behave in independent generate both anther and pollen development (Millar & Gubler, 2005). Recent findings have suggested that MYB genes are post-transcription structured through microRNAs; examples include *AtMYB33, AtMYB35, AtMYB65* and *AtMYB101* genes, which are involved in the development of the antherand pollen targeted by miR159 family (Allen *et al.*, 2007). Gibberellins (GAs) too have a significance role in anther development. GAMYB that is a transcription factor is also part of the processes of the GA-regulation of the expression of the genes within the anthers (Kaneko *et al.*, 2004; Tsuji *et al.*, 2006; Aya *et al.*, 2009). Similarly speaking, the plant hormone jasmonic acid, as well as its derived chemicals which are referred to as jasmonates remain the key regulating factors of the development of stamen, as well as the maturity of the pollen.Three of the MYB transcription factors *MYB108, MYB21* and *MYB24* are comprised in the jasmonates response within the stamen. Analysis of plants with insertions within these genes demonstrate a lower male fertility which is connected to

the delay in the anther dehiscence, as well as reduced pollen viability, and therefore a decrease in reproductive ability (Mandaokar *et al.*, 2006; Mandaokar & Browse, 2009). Additionally, the MYB proteins are also seen to be included within the female fertility ability. The *AtMYB98* has control over the difference of the plant's synergids cells, thought the development of the gametophyte a well as the mutant *myb98*, which are synergised cells that possess defect characteristics within their filiform apparatus as well as pollen tube guidance (Punwani *et al.*, 2008). Within the male gametophyte mutations seen in the male germline, the *AtMYB125/DUO1* can be seen as the initial germline which is specific with regard to the R2R3 MYB transcription, and this is demonstrated to be a key indication of the division of the cells, as well as the differences within the cells (Durbarry *et al.*, 2005; Rotman *et al.*, 2005; Brownfield *et al.*, 2009a).

1.6.2. C_2H_2 Zinc Finger Transcription Factor Family in plants

DAZ1 and DAZ2 are members of the family of C_2H_2 zinc finger proteins (ZFPs), which comprises the largest number of transcription factors in *Arabidopsis thaliana*, according to the recent count based on *in silico* analysis (Englbrecht *et al.*, 2004). What defines this family of genes is that members possess one or multiple C_2H_2 zinc finger domains (Takatsuji, 1999). Made up of 25-30 amino acid residues, these domains exhibit at both ends two cysteine or histidine residues, which tetrahedrally manipulate a zinc atom to produce a compact finger structure that displays a β-hairpin and α-helix. As demonstrated by structural research of crystallised zinc finger-DNA complexes, such as the one conducted Choo & Klug, (1997), there is direct contact between the α-helix and the primary DNA groove. Brown, (2005) provided evidence that DNA and/or RNA are bound by C_2H_2 ZFPs. Moreover, these domains have been noted to be actively involved in interactions between proteins (Brayer & Segal, 2008). Isolated from *Petunia*, ZPT2-1 is the first C_2H_2 ZFP to be detected in plants (Takatsuji *et al.*, 1992).

After these research studies ended in the identification of other zinc finger proteins are present in during the flower development .For instance of this is the SUP (SUPERMAN) that continues the division of the first and the fourth whorls of flower (Sakai *et al.*, 1995). In the case of the SUP-protein such as RBE (RABBIT EARS), was demonstrated to be an important role in the development of the petals and the

continuation of the boundaries of the homeotic gene expression that is identified between whorls (Krizek *et al.*, 2006).

Several zinc finger proteins have also been noted if they are redundant in their effect such as NUMBIN(NUB) that is responsible for JAG redundancy. It does not act in a meaningful manner to JAGGED(JAG) which is C_2H_2 TF ,in the promote of the development of the pollen-bearing microsporangia of the carpel walls and the anthers of the gynaecium (Dinneny *et al.*, 2006). DAZ1 and DAZ2 are 2 of 60 C_2H_2 TFs expressed during male gametophyte development (Honys and Twell, 2004). The C_2H_2 family of TFs are common to animals and plants and many are thought to be DNA-binding proteins. They have at least one zinc finger domain of ββα conformation, the alpha helix being made up of pairs of the cysteine and histidine amino acids responsible for zinc atom coordination. It is α helix that binds to the DNA major groove, having specificity for a 3-nucleotide sequence (Isernia *et al.*, 2003). In *Arabidopsis* C_2H_2 TFs are classified based on the number and spacing of zinc fingers and the number of amino acids between the zinc-coordinating histines (Englbrecht *et al*., 2004). DAZ1 and DAZ2 have dispersed zinc fingers, placing them in class C, and subclass C1 because the 3 amino acids are between the histidines. They are 2 of 8 members of the subclass C1-3i, having 3 zinc fingers. The 6 amino acid sequence of the α helices of the zinc fingers proximal to the pair of histidines place DAZ1 and DAZ2 in subgroup C1-3iC (having the sequence KALFGH, QALGGH and QALGGH for the first, middle and end finger fingers, respectively (Englbrecht *et al*., 2004). Furthermore, a majority of the members of this grouping also have the same putative, nuclear localisation sequences in addition to their repression-domains. This repressive action is mediated through the EAR motif, (Ethylene-responsive element binding factor-associated Amphiphilic Repression) (Englebrecht *et al*., 2004). In accordance with recent analysis of EAR that included transcriptional elements the C_2H_2 family, as the largest of repression motif that contains protein strains. Indeed, the EAR motif locations are found within the C-terminal area, but can be also seen in the N-terminal and the middle area of the protein sequence (Kagale *et al.*, 2010).

Transcriptome data suggest expression of DAZ1 and DAZ2 to be pollen-specific (Pina *et al*., 2005; Honys and Twell, 2004), which has been confirmed by RT-PCR on RNA from dehisced pollen and the sporophyte (Borg *et al.*, 2011). The degree of DUO1

transactivation was measured by conducting ATH1-based gene expression profiles of *Arabidopsis* seedlings after DUO1 ectopic expression to determine DUO1 targets (Brownfield *et al.*, 2009a). Also, employing available data on *Arabidopsis* sperm cell transcriptome to screen for male gametophyte-present transcripts (Borg *et al.*, 2011). A paralogous set of C_2H_2 zincs finger DUO1-Activated Zinc finger (DAZ) proteins; DAZ1 (At2g17180) and DAZ2 (At4g35280) were among the 63 presumed DUO1 targets. DAZ1 and DAZ2 promoters, when driving H2B::GFP, showed germline-specific activity and qRT-PCR has shown that levels of DAZ1 and DAZ2 mRNA rise from BC, peaking in TC pollen before decreasing in MP (Borg *et al.*, 2011). DAZ1 and DAZ2 represent a subset of DUO1-activated zinc finger transcriptional repressors that are functionally redundant and specifically express in the germline. Also, a new study has emerged that demonstrates the importance of a pair of DUO1-dependent C_2H_2 zinc finger proteins called DAZ1 and DAZ2 that are required to promote germ cell division and gamete differentiation in *Arabidopsis* (Borg *et al.*, 2014). Molecular origins of male gametophyte development in Angiosperm are not well understood and at the molecular level, excluding *Arabidopsis thaliana*, male germline development in flowering plants has not been investigated empirically in depth. The continuing publication of plant genome sequence data is facilitating the identification of homologs of important *Arabidopsis* regulatory factors critical for pollen development.

1.6.3. Germ cell division and specification: DUO1 the master regulator

DUO1 is a male germline-specific R2R3-type MYB transcription factor that is initially expressed in the newly formed generative cell or male germline following asymmetric division of the microspore (Rotman *et al.*, 2005). The mutant *duo1* cells did not enter the PMII, though this does not mean that the germ cell is therefore prevented to go into its S stage (Rotman *et al.*, 2005). The DUO1 encoded a couple of tryptophan-rich repeats (R2R3), that had DNA binding locations which are rendered to as MYB domains, these bind the significant groove of the target-DNA though the helix-turn-helix structure that interacts with both bases and phosphates, in accordance with Saikumar *et al.* (1990).

Additional with RT-PCR analysis showed that DUO1 transcription peaked at the bicellular pollen stage, thought which the male gametogenesis was found to be germ cell mitosis, and this validated its status as a significant germline-specific regulation, which was needed for the transition of the G2/M. Furthermore, the failure of the *duo1* cell, which failed to enter the stage of mitosis, is generated through the absence of the lack of core cells, of the cycle component *CYCB1;1* more exactly, those that are needed at the G2/M transition, which indicates that the *CYCB1;1* is, indeed a DUO1 dependant one. Besides the function-based characterises of the duo1 mutations show that the mutated germ cells were actually not able to fertilise, and this indicates that the *duo1* does not merely create cell-cycle-defects, but additionally does not have the needed features required for gamete differentiation (Brownfield *et al.,* 2009a). It has nevertheless been shown that the DUO1 is a regulator for the germline specific gene that is inclusive of MGH3 (*MALE-GAMETE-SPECIFIC HISTONE H3*) which encodes a germline specific histone of H3.3 variation (Okada *et al.*, 2005), *the GEX2* (*GAMETE EXPRESSED 2)* which is encoded in a putative membrane which is associated with the relevant protein furthermore the (Engel *et al.*, 2005), and *GCS1 (GENERATIVE CELL SPECIFIC 1)* encoding the sperm cells surface protein that is needed for the process of fertilisation (Mori *et al.*, 2005). The targets promoter within the *duo1* mutant drive assessment of the GFP expression, and this have shown to demonstrate no expression or the weaker GFP signal, within the mutant germ cells. The absence of expression of the *GCS1* within the *duo1* cell ends in the infertility of the mutant pollen of the same, which shows that the activation of the DUO1 is also a suppressing expression if the *GCS1*m indicating that the activation of the *GSC1* is dependent upon the DUO1. These findings demonstrate that the DUO1 is featured as a role within the male germline developmental process, and this is needed for the germ cell division, as well as the germ cell specification in order that they are then able to create functioning sperm cells afterward (Brownfield *et al.*, 2009a). Additionally, the DUO1 was expressed in ecotypical terms within seedlings with the use of a resistant DUO1 cDNA (mDUO1) including an alteration of the nucleotide sequence at the microRNA159 site that is used for binding. An inducible promoter estradiol was employed for the purpose of driving the expression of mDUO1 within the sporophytic tissues. Inducing the mDUO1 was also found to yield an insufficient amount for the CYCB1; 1 transcript (Brownfield *et al.*, 2009a).

This means *DUO1* is able to do and thus activate targeted estradiol induced m*DUO1* constructs (Zuo *et al.*, 2000). This methodology was employed with the intention of noting the additional targets of regulation of *DUO1*. The findings of this study showed that 63 of the putative targets had a high value of fold increase expression following their induction of DUO1. From these fourteen of the target genes were demonstrated to be regulated by the DUO1, with it binds to a canonical MYB site in the case of target promoters (Borg *et al.*, 2011). Concerning the DUO1 targets such putative targets show that they represent many gene families inclusive of the germline enriched genes, which are, needed for the development of the germline and their specification.

DUO3 forms an additional positive germline regulatory factor that, similar to DUO1, is needed for the division and the specification of the sperm cells. The DUO3 has the same regulatory target as the DUO1, and this is also included in the activation processes of *GCS1* and *GEX2*. It has furthermore been demonstrated that in the case of there being non-DUO3, the ordinary expressive state of the germline *GCS1* and *GEX2* are suppressed as a result. Nevertheless, the germline expression is the DUO1 dependant gene, which is independent of that of the DUO3 activation. The expression of the *CYCB1;1* continues within the *duo3* cell, though not in the case of the *duo1* cell, which indicates that the DUO3 encourages mitosis entry independent of *CYCB1;1*, which is dissimilar to DUO1. Despite this, mechanisms of this couple of germline regulators are used to stimulate and activate normative targeted genes throughout the development of the germline (Brownfield *et al.*, 2009a), though this still remains unknown. In addition to this, the *in silico* assessment of the *DUO1* gene has shown that the sequential order shares high levels of identification with the nucleotides that match the MYB location of the eleven different *DUO1*-like proteins, which has been discovered to be evolutionarily retained within seven extant plant families, as well as two of the main clades monocots and eudicots (Haught, 2007). The *DUO1* homologues are also seen to exist within bicellular (*Nicotiana tabacum*) and tricellular (rice, maize and *Arabidopsis*) species though it is also seen within some non-flowering plant such as *Physcomitrella patens* and *Selaginella moellendorfii* (Brownfield *et al.*, 2009b). Since the regulation of generative cell division may have been a significant aspect of the heterochronic shift from tricellular pollen, functional conservation of the DUO1 orthologs, suggests an important role in the evolution of bi- and tricellular species (Rotman *et al.*, 2005).

1.7. Selected species for comparative analyses

1.7.1. Tomato as a model plant

Tomato is cultivated globally and forms a large staple of the agricultural produce of the world. After the potato, the tomato is the second most globally consumed vegetable, and is potentially the most popular domestic crop in the world. Within the US, the tomato is the third most important commercial vegetable of a total $2.062 billion is value after the potato and lettuce crops (which are worth $2.564 billion and $2.064 billion respectively). Furthermore, tomatoes consumed either raw or cooked, and they can be used in the production of other foods or in different mediums like past or soup, indeed tomato juice is even consumed. The primary tomato-crop-producing nations are China, the USA, India, Egypt, Turkey and Italy (http://faostat.fao.org). The tomato as it was cultivated which named as *Solanum lycopersicum* by Linnaeus (1753). In the year of 1754 Miller distinguished the tomato plants and dubbed each type to the genus *Lycopersicon* and the species *esculentum* in the case of the cultivated plant (Miller, 1754). The phylogenetic connection between these two plant names has remained a contentious issue, with many arguing that the *Lycopersicum* plant is, in fact, a different species, while others indicate that the species needs to be lumped into the *Solanum* species. In Contemporary times, the tomato has been founded in the molecular and morphological data connected to the plant, and new taxon for the tomato and the read choice of the *Solanum lycopersicum* in the case of the cultivated tomato plant have been argued (Knapp *et al.*, 2004; Olmstead *et al.*, 1999; Peralta & Spooner, 2001). Regardless, the tomato plant *Solanum lycopersicum* is found in the *Solanaceae* family, which contains almost 2,500 species and over 100 genera. Within this plant family, there are notable numbers of agriculturally useful species such as the aubergine, the potato, the pepper, and tobacco. The tomato has been selected for the purpose of genetic sequencing due to the fact that they are generally the most intensively farmed of all the relevant plants and the most genetically characteristic. The diploid genome (n=12) of the plant of *S. lycopersicum* has a reasonably elementary architectural structure, being around 950Mb in size. With regard to all crop species, those in this family remain the most useful and therefore have such a diverse agricultural utility, and they are the third with regard to their commercial characteristics, which are only transcended by legumes

and grasses in value. The tomato possesses a superlative modelling system with regard to both the basic and the applied plant research. This is as a result of several reasons, (Rick & Yoder, 1988) which is inclusive of; ease-of-culture within various environments; a short life cycle a short life cycle; photoperiodic insensitivity; simplicity controlled pollination as well as hybridization; it is a diploid species with a small genome (Peterson *et al.*, 1998) is capable of whole plant regeneration (McCormick *et al.*, 1986; Fillatti *et al.*, 1987) and lacks gene duplication, as well having asexual propagation amenability; the ability to develop haploids (Zagorska *et al.*, 1998), and the means to a wide array of mutants (Menda *et al.*, 2004) genetic stocks. Form the time of the realisation of the high-accuracy sequential assessment of an entire genome of the species *Arabidopsis thaliana* in 2000 by *Arabidopsis* Genome Initiative, all of the nucleotide sequences which include the totality of a plant species or else near-complete elements of a genome sequence has been published for several species of plants inclusive of the rice plant (by the IRGSP in 2005), the grapevine, *Lotus japonicus*; (Sato & Peet, 2005) and the poplar (Tuskan *et al.*, 2006), among others. The international Tomato Sequencing Project in 2003 was devised by members from ten various nations, with the intention of sequencing the genetically gene-abundant areas of twelve of the chromosomes with the use of a high-quality sequencing of bacterial chromosomes artificial (BAC), selected on the basis of DNA markers that are found within the genome, according to Mueller *et al.* (2005). Across the last decade, great attention and action has been given to the development of genetic and genomic means with regard to the tomato plant, inclusive of genomic clones, DNA markers and genetic maps, mapping of populations and genetically connected link-maps. These means permit the exploration of the genomic structure and the functions of all of the genes as well as the identification of the genes, which are of notable agronomic significance. In a parallel, internationally-based projects including the participation of 90 grouping from a total of fourteen nations have been trying to use these resources to go forward with the intention of analysing and deciphering the genetic information that is found in the tomato plant.

With the assistance of innovative sequencing means and the employment of high-quality bioinformatics, the sequencing of the total genome of the tomato plant was published in May 2012 (The Tomato Genome Consortium) (Sato *et al.*, 2012). Accompanied by advances that have been made in genomic studies, significant portions

of the sequence information (for instance, over 200,000 expressed genetic tags (ESTs) and a total of 90,000 BAC-ends have been noted for the tomato plant with the use of the SGN network (Sol Genomics)). This is a recognised community effort in the world of genetic and genomic research within *Solanaceae*, according to Mueller *et al.* (2005). SNPs have been noted on the basis of the EST sequences in the case of several tomato cultivation areas; such as (Yamamoto *et al.*, 2005; Aoki *et al.*, 2010). Two population maps have been produced from crosses between *S. lycopersicum* or 'micro-tom' and *S. lycopersicum* 'Ailsa Craig' or the 'M82' *S. lycopersicum*, all of which were given a linkage analysis of SNP markers. Throughout the following years, many, indeed, hundreds of *Solanaceae*-based genetic frameworks inclusive of phenotype and sequences of one hundred genomes, included the phylogenetically diverse nature of the group as a whole. This scheme, referred to as the SOL-100 network, includes the sequencing of one hundred differing *Solanaceae* genomes, and the links of these sequences to that of the referenced tomato sequence in the intention of trying to attain an exploration into the significant issues within plant life and its diversity, as well as the conservation of genomic diversity.

1.7.2. Rice as a model plant

As a monocotyledonous angiosperm, rice belongs in the genus *Oryza*, which includes in excess of twenty other plant species. Two of these are seen as true cultivated rice plants; *Oryza glaberrima*, which was originally cultivated in the West of African continent, and *Oryza sativa* (Watanabe *et al.*, 1997) which originated within South-east Asia and the Japanese mainland. Rice was first cultivated in the tropical zone of Asia, and the most ancient recording of its cultivation dates to 5000 BC, after which its growing dissipated into more temperate areas of the continent (Watanabe *et al.*, 1997). The crop remains one of the most sustainably consumed and grown within the Asian continent, and in excess of nine out of ten of all rice products is grown in the Asian continent (where six out of ten people of the global population live). Indeed thirty-five to sixty percent of all calories required by three billion Asian people are constituted by rice (Guyer *et al.*, 1998). In excess of 150-million hectares of rice are planted each year, which accounts for nearly a tenth of the global land available for crop growing, which, in 1999-2000, surmounted to a to a of 0.6 billion tonnes of seed being produced (or 386 million tonnes

once the rise was milled). Considering that the global population is set to grow from 6.2-8.2 billion from 2000-2030, requirement for the production of rice itself will grow to a total of 765 million tonnes (533 million tonnes after milling (FAO, 2002). Following the green revolution almost thirty years ago, the annual production of rice has swelled by roughly 2.5 percent per annum. Throughout the 1990s, this fell to a mere 1.1% (Nanda, 2000). Save for its continuing economic status throughout this time, rice has also maintained its status as within both genomic and genetic academic research. As a diploid with twenty-four chromosomes (2n=24), rice's chromosomes can be separately noted and understood through the use of cytogenic assessment, according to Fukui & Iijima (1991). The genome that belongs to rice is not large, (at roughly 430Mb), which is dwarfed by the size of other crop genomes like maize, at 2,400Mb; wheat at 16,000Mb; and barley at 4,600Mb—the genome includes 32000-64,000 genes in total (Sasaki & Sederoff, 2003). Consequently, rice, with its relatively small size of genome has ensured that rice is utilised as a notable model for the assessment of cereal genomics in addition to being a general model for monocotyledonous plant. Academic research into the production of rice has created several notable tools that have been used in the genetic analysis of the plant itself, for instance the creation of high-density molecular genetic maps as well as proficient genetic transformational techniques (Hiei *et al.*, 1997). Comparing these genetic maps with that of other species within the grass family suggests that there is gene conservation present as well as genetic ordering of genes (synteny) within the genomes of itself (Ahn & Tanksley, 1993; Devos & Gale, 1997; Devos *et al.*, 2000). The Beijing Genomics Institute (BGI) (Yu *et al.*, 2002) and the Syngenta's Torrey Mesa Research Institute (TMRI) (Goff *et al.*, 2002) have released drafts of the rice genome sequence for two rice subspecies. A continuing endeavour has been taking place under the supervision of the IRGSP (International Rice Genome Sequencing Project), who are trying to structure a total high-quality draft of the genome of rice, and which to date suggests that it will be a useful tool within the rice breeding and research communities (Sasaki & Sederoff, 2003; Sakata *et al.*, 2002). Molecular markers are being employed to discover the genetic map of rice, such as RLFP (Kishimoto *et al.*, 1993), as well as Simple Sequence Repeats (SSRs), as used by (Temnykh *et al.*, 2000; Wu *et al.*, 2002) have been utilised. Roughly seventy percent of the maps using RFLP have been devised with the use of cDNA probes too, and of this number, almost thirty percent possessed a notable sequential homology to that of

presently known genetic sequences (Kishimoto *et al.*, 1993). Physical maps that have been constructed of the genome of rice have been structured on the basis of ESTs, Sequence-Tagged Connectors (STCs), bacterial artificial chromosome (BAC), yeast artificial chromosomes (YAC), P1-derived artificial chromosomes (PAC), or shotgun sequence analysis. Rice ESTs, which 68,000 are found in public information database and the rest are found privately, database (Tarchini *et al.*, 2000). Of these members however, a mere quarter have actually demonstrated a significant and notable homogeny to those of known genes, and thus the point of the most genes remains uncertain (Jeon *et al.*, 2000). As a greater proportion of the rice genome is mapped, it becomes more and more important to see the connection between thousands and thousands of rice genes. Conversely, the reverse genetic methodology tries to achieve this through the comparison of the sequence and its similarity between different plants with the use of the EST markers. The initial rice gene that was cloned with the use of map-founded cloning process was seen to be a *Xanthomonas campestris* cv. *Oryzae resistance* (Xa21) gene by Song *et al.* (1995), as well as Xa1 by Yoshimura *et al.* (1998). Generally speaking, rice mutations may be generated through the use of either chemical or else physical mutagens like EMS (ethyl methane-sulphonate), as used by Inukai *et al.* (2000) and Goel *et al.* (2001), as well as gamma-ray irradiative techniques, which are a more traditional means still used by some (Teraishi *et al.*, 1999; Biswas *et al.*, 2003). Others use an insertional mutagenesis of the sequence through transposing elements of the DNA and using T-DNA of the mediated transformation of *Agrobacterium tumefaciens*, as used by Izawa *et al.* (1997) and Jeon *et al.* (2000). The employment of this later technique allowed for an increase in the efficiency of the transformational method developed for rice (Hiei *et al.*, 1997). As this process was achieved, the outcomes of the technique remains somewhat uncertain, (Izawa *et al.*, 1997; Jeon *et al.*, 2000; Jeong *et al.*, 2002), and though the method has its benefits over that of alternative techniques, it requires a noting of the molecular and genetic code as well as the insertion of marker genes which are carried within the insert (Martienssen, 1998).

1.7.3. Moss as a model plant

Bryophytes comprise hornworts, liverworts and mosses. With regard to its phylogenetic

significant position in the scope of a diverging land-based plant which connects a period of nearly one billion years from the unicellular green algae stage to that of flowering plants *Physcomitrella patens* (the moss) combines a great deal of the benefits that are needed for a model organism. Mosses show alternation of generations with a dominant haploid gametophytic generation. The two generations are distinct from one another in terms of morphology and DNA content. A moss gametophyte develops in two phases. The juvenile filamentous form called protonema, which grows by apical cell division and the adult form or gametophore stage, which grows by division of three faced apical cells analogous to that of higher plants (Reski, 1998). The predominant haploid phase together with high rate of homologous recombination in moss DNA at higher frequencies than in land plants makes it an efficient system for using both reverse and forward genetic techniques to study gene functions related to almost all aspects of plant biology. These studies may help in understanding the role played by various male germ line regulators in evolution of double fertilization in higher land plants. Mosses are simple land plants, with relatively few cell types and their organs consist of single cell sheets. Their simple morphology has made the study of developmental processes in plants easier. It requires very simple growth conditions to complete its life cycle.

It can be grown under axenic conditions on inorganic media devoid of any phytohormones or vitamins using *in-vitro* plant tissue culture techniques (Reski & Abel, 1985). Mosses have been used to study plant development, using genetic and physiological procedures, for over 80 years now. Von Wettstein's research group, from 1920-1945, realized the importance of moss as an important model organism and was at the forefront of genetic research at that time (Cove *et al.*, 1997). The genetic techniques for moss transformation were first developed using polyethylene glycol-mediated DNA uptake by isolated protoplasts and the method is almost exclusively applied (Schaefer *et al.*, 1991). *Physcomitrella* is the only plant that undergoes homologous recombination with a frequency that allows easy targeting of genes for replacement and elimination. This unique feature allows precise manipulations of the genomic DNA by gene replacement using suitable gene disruption constructs (Schaefer & Zrÿd, 1997). The gene disruptions in haploid moss results in immediately visible and genetically stable mutant phenotypes because of absence of second allele. The reduced gene redundancy found in *Physcomitrella* is another advantage for gene-function analyses as the

disruption of a gene is more unlikely to be complemented by another member of the same gene family. The first knockout mutant of moss clearly showing altered phenotype was obtained by targeting of the *Ftsz2-1* gene (cell division protein), which resulted in mutant with only one big macro chloroplast per cell, thus clearly identifying *Ftsz2-1* as first organelle division protein in eukaryotes (Strepp *et al.*, 1998). The single gene knockouts in moss nuclear genome have become efficient due to high rate of homologous recombination found in *Physcomitrella patens* and is achieved by transformation of *Physcomitrella patens* protoplasts with DNA fragments containing the gene of interest that has been disrupted by the integration of selection marker cassette. These knockout constructs can be generated using cDNA or genomic DNA, with suitable restriction sites, located approximately in the central region, for the integration of selection marker cassette. Over the past twenty years, an expanding community has set up the *Physcomitrella patens* as a model organism that is in possession of a well-developed toolbox of molecular benefits inclusive of the uniquely proficient genetic targeting through the homologous recombination and the inclusion of comprehensive genetic resources that are now available through the use of the central web service cosmoss.org (Lang *et al.*, 2005).

The draft genome sequence was made public in 2008 (Rensing *et al.*, 2008). It is also the first bryophyte genome to be sequenced. Its availability, in addition to the established molecular toolbox, permits a broad comparison in evo-devo investigations. This can be seen in the expanding record of publications using *Physcomitrella* as an additional model organism in comparative studies (Lang *et al.*, 2005; Hirano *et al.*, 2007). Many evo-devo studies have shown that *Physcomitrella patens* orthologs can complement in an *Arabidopsis* mutant background (Yasumura *et al.*, 2005; Perroud & Quatrano, 2008).

1.8. Aims and Objectives

1) The first major objective of this thesis, presented in Chapter 3, was to assess the validity of Brewbaker's evidence that Magnoliaceae family species possess exclusively bicellular pollen at anthesis and to explore the effect of temperature on pollen cell division.

a) analysis of pollen from herbarium and living specimens of forty-one *Magnolia* species. These were sourced from the Natural History Museum herbarium and various botanical gardens from United Kingdom, United States, and China.

b) examination of whether temperature could affect the rate of division of the generative cell. The number of pollen nuclei was scored in flowering branches of *M.grandiflora* cultured under different temperature regimes in controlled growth chambers.

2) The second major objective was to investigate functional conservation of DUO1 in plants. The results of this investigation, described in chapter 4 test the function of DUO1 protein in different angiosperm species.

a) to identify expression of DUO1 protein in selected plant tissues using total RNA and RT-PCR.

b) the design of a transient luciferase assay was employed to quantify transactivation potential of DUO1 proteins.

c) complementation potential analyses of the DUO1 variants in selected species, with the help of an in vivo pollen assay.

d) a corroborative experiment looking into the biological function of complemented *duo1* germ cells (male transmission analyses) is examined in *Arabidopsis*,tomato and rice.

3) The third major objective was to investigate the functional conservation of *DAZ1/DAZ2* zinc finger proteins between the dicot species, *Arabidopsis thaliana*, *Solanum lycopersicum*, *Brassica rapa* and the monocot temperate grass model, *Brachypodium distachyon*.

a) the identification, cloning and sequencing of the predicted *DAZ1*-related sequences from genomic DNAs of the selected species.

b) complementation potential analyses of the *DAZ1* variants in an *in vivo* functional assays

c) investigatation of plant fertility in *daz1,daz2* double mutants harbouring a homozygous *DAZ1*-transgene from a heterologous species.

Chapter 2

MATERIAL AND METHODS

2. Material and Methods

2.1. 2.1. Purchase Material

Materials and chemicals were bought from the following sources: Melford Laboratories; DHAI PROCIDA; PerkinElmer; BioGene; Promega; Duchefa Biochemie; Calbiochem; Lehle seeds; Sigma-Aldrich and Thermo Fisher Scientific. All purification kits were bought from Qiagen, Bioneer or Sigma-Aldrich. Additional reagents and enzymes required were obtained from: Bioline; Novagen; New England Biolabs; Invitrogen and Sigma-Aldrich.

2.2. Plant propagation and tissue culture

2.2.1. Media for plant tissue culture

Arabidopsis seeds were plated on Murashige and Skoog salt media (Duchefa Biochemie). 4.3g of MS salt were dissolved in 1000 ml of distilled water with no added sucrose (MSO). The pH of the media was adjusted to 5.8 with 5N NaOH and autoclaved for 15 minutes at 120 ^{o}C and 15 psi on liquid cycle. To prepare MS agar for plating, 0.6 % (w/v) of Phyto Agar (Duchefa Biochemie) was added prior to autoclaving.

2.2.2. Seed sowing and growth conditions

Arabidopsis thaliana plants of ecotype Columbia (Col-0), and Nossen (No-0) were grown in soil of a 3:1:1 ratio of Levington F2+S compost (Skotts, UK), sand and vermiculite respectively. Seeds were sown onto soil and placed at 4 ^{o}C for two days before being moved to growth room. Room condition was 22 ^{o}C with continuous light and variable humidity. When seedlings had developed two cotyledons they were transferred to individual pots.

2.2.3. Seed surface sterilization and plating on media

All F1 generation seeds were sterilized with 70% (v/v) ethanol (including 0.5% Triton X-100) for 5 minutes, then, they were incubated in 100% (v/v) ethanol for 1 minute. The seeds were placed on filter paper to dry for 15-20 minutes. For plating F1 seeds, 50-100 seeds were plated on MS0 media with suitable antibiotic selection. For selection of PPT resistance, seeds were selected on MS0 medium with 10 mg/ml DL-Phosphinothricin. Wild type seeds were plated on MS0 media without antibiotic resistance. Plastic petri dishes were sealed with ventilated tape and kept at 4^{o}C for two days, and then incubated in the growth room set at 22^{o}C with variable humidity and continuous light.

2.2.4. *Pyhscomitrella patens* (Moss) culture

2.2.4.1. Media for Moss tissue culture

BCD media is used for routine culture of moss as it promotes rapid growth. In homogenate cultures, the tissue will be an almost exclusively chloronemal filaments for the first week. Caulonemal filaments will differentiate and buds will form giving rise to gametophores. Diammonium tartrate should be omitted for culture of plants then reproductive organs and sporophyte development is desire. Subcultures were transferred to BCD media without diammonium tartrate to grow sexual parts and sporophytes, they were kept at 15 ^{o}C 8 hours light 16 hours dark condition in the growth chamber.

BCD media

Reagents	Volume
Stock solution B ($MgS0_4.7H_20$)	10 ml
Stock solution C (KH_2PO_4)	10 ml
Stock solution D (KNO_3)	10 ml
Trace element solution (TES)	1 ml
Phytoagar	5.5 g
Distilled water	Up to 1 litre

The mixture was sterilized by autoclaving. If needed diammonium tartrate (5mM) should be added before autoclaving. 1M $CaCl_2$ was added in the media before pouring petri dishes. Moss colonies were transferred from previous colony gametophytes to make fresh subculture colonies under sterile conditions. Also, a sample of the same individual colony was added to 5ml LB media in a universal bottle to analyse bacterial contamination.

2.2.4.2. Moss growth condition

For routine culture of moss tissue, BCD media was used with 5mM diammonium tartrate and 1M $CaCl_2$, solidified with agar to obtain vegetative tissue. Initiation of reproductive tissues involves growing the moss on a slightly nutrient-deficient medium. BCD media was used containing 1M $CaCl_2$ without ammonium tartrate. The trick is to grow the moss until a large colony with healthy gametophores (leafy shoots) is formed. Then it is transfered it to low temperature/sort day conditions. After about two weeks, the moss should have developed sexual organs, the archegonia and antheridia. The culture was irrigated with sterile water to provide the sperm with a medium in which to swim. This was done by pouring a small volume (2-3 ml) water over the leafy shoots leaving in place for a few hours, then pouring off the excess liquid. It was done once a

week, until spore capsules developed. When spore capsules they turned brown, they were harvested and dried to grow fresh generation.

2.2.5. Antibiotics for selection of transgenic plants

Antibiotic	Working concentration mg/l
Kanamycin	50
Hygromycin	20
Gentamicin	100
Phosphinothricin	10
Glufosinate (Basta)	30

2.2.6. Storage of plant tissues

All collected plant tissues (leaves, sepal, petal, stamen, carpel and pollen) for RNA and DNA isolation were weighed, flash frozen in liquid nitrogen and stored at -80^{o}C.

2.3. Bacterial culture and storage

2.3.1. Bacterial strains

E. coli	*A. tumefaciens*
α-select (Bioline)	
DH5α	GV3101

2.3.2. Preparation of chemically competant *E. coli* cells

In accordance with the criteria stipulated by (Hanahan, 1983), *E. coli* DH5α were grown on an LB agar plate and a single colony of was grown overnight in a volume of 25ml of SOB medium containing; 0.5% Yeast extract; 10mM $MgSO_4$; 10mM $MgCl_2$; 2 percent bactotryptone; 2.5mM KCl; 10mM NaCl at a temperature of 37^oC at 200 rpm. A 1ml aliquot was inoculated into 100ml of pre-warmed LB, including tetracycline and grown at 30^oC until the OD_{600} reached 0.2-1M, after which $MgCl_2$ was included into the mixture at a concentration of 20mM (ie; 2ml of 1M $MgCl_{2)}$ and then the cells were grown until the OD_{600} reached 0.45-0.55.

The bacteria were transferred to a 50ml test-tube that had been previously sterilised before being kept on ice for 20 minutes. The mixture was then placed into a centrifuge at a temperature of 4 ^oC and centrifuged at 500 x g for a period of five minutes. The pellet was re-suspended in 50 ml $Ca^{2+}Mn^{2+}$ solution containing 40mM sodium acetate, 70mM $MnCl_2$ and 100mM $CaCl_2$, pH5.5 (adjusted with 1M filter sterilised HCl) and incubated for 45 minutes. The cells of the bacteria were then placed in a centrifuge at five thousand revolutions per minute for a period of five minutes, after which the supernatant was discarded and the pellet was re-suspended in 5 ml $Ca^{2+}Mn^{2+}$ solution which contained 15 percent v/v glycerol. Finally, 200µl of the cells were aliquoted into 1.5ml test tubes, along with liquid nitrogen, to be kept at a temperature of 80^oC.

2.3.3. Preparation of chemically component *A. tumefaciens* cells

A strain of *Agrobacterium* strain (GV3101), align with a 'helper' T_i plasmid was developed in 5ml of LB media, which contained 50mg/ml of Rifampicin overnight held at a temperature of 28^oC. Shaking of the mixture was also conducted at 200 rpm. 2ml of the aliquot overnight culture was put into the 50ml of the mixture of the LB medium win a flask of 250ml capacity. This culture was then developed at a temperature of 28^oC with significant shaking of 250rpm unto the point at which the OD_{6000} hit a level of 0.5-1.0. This liquid suspension was then cooled on ice and placed in a centrifuge at 3kg for a ten-minute period at a temperature of 4^oC. All of the supernatant was thrown away and the cells re-suspended within the 1ml of 20mM ice-temperature $CaCl_2$ liquid. 100µl

of the cells were expediently allocated into the formally cooled microfuge tubes and then 'flash-frozen' in liquid Nitrogen before being kept at a temperature of 80^{o}C.

2.3.4. Antibiotics for bacterial selection

Antibiotic	**Working concentration mg/ml**	
	E.coli	*A. tumefaciens*
Ampicillin	30	30
Chloramphenicol	30	20
Kanamycin	50	50
Rifampicin	Na	50
Spectinomycin	100	100

2.3.5. Long-term storage of bacterial strains

300 µl sterile 50 % (v/v) glycerol and 700µl of an overnight bacterial culture were mixed and placed in a cryogenic storage tube. Before storing mixture was frozen in liquid nitrogen at -80^{o}C.

2.3.6. Transformation of competant *E. coli* with plasmid DNA

25µl aliquot of cells was taken from – 80^{o}C freezer and thawed on ice for 10 minutes. 1µl of plasmid or half of a ligation reaction was added into a 25µl aliquot and incubated on ice for 30 minutes. The mixture was replaced in a water bath at 42 ^{o}C for 45 seconds to heat shock, and then the mixture was transferred back on ice for 2 minutes. 950µl LB media was added and the culture was incubated at 37 ^{o}C for one and a half hours in the orbital shaker (Sanyo Gallenkamp, UK) at 220 rpm. A 100 µl of bacterial cultured cells

was plated on LB agar, which contain selected antibiotics. Cultured cells plates were incubated at 37^{o}C overnight to grow bacterial colonies.

2.3.7. Transformation of competent *A. tumefaciens* with plasmid DNA

Between 0.5-1 μg of plasmid DNA was added in the frozen aliquot and incubated at 37 ^{o}C for 5 minutes to heat shock the cells. The cells was mixed into the 1ml LB media and then incubated at 28 ^{o}C at 200 rpm in the orbital shaker for between 2-4 hours. The mixture was centrifuged for 30 seconds at 5000rpm and half of the supernatant discarded. The cells were re-suspended with remaining solution and plated on LB agar plates, which contain selected antibiotics. The plates were incubated at 28 ^{o}C for two days to grow colonies.

2.4. Nucleic acid extraction

2.4.1. Single leaf genomic DNA extraction for genotyping

One-two leaves were collected into a 1.5ml eppendorf tube, which contain 200-300 glass beads (Sigma-Aldrich) with a size of 425-600μm for genomic DNA extraction. The leaf tissues were frozen in liquid nitrogen and ground for 12 seconds in a Silamet amalgam mixer (Ivoclar Vivadent, UK) at room temperature. 250μl extraction buffer was put in the eppendorf tube and vortexed well and incubated for 15 minutes at room temperature. 250μl chloroform : IAA (24:1) was added, then mixed well by vortex and centrifuged for 12 minutes at 14000rpm. The upper aqueous phase that approximately 200μl was transferred into a new eppendorf tube containing a 0.7 volume approximately 140μl of isopropanol. The mixture was gently shaken 4-5 times by hand. After that, the mixture incubated for 5 minutes at room temperature and centrifuged for 7 minutes at 14000rpm. The supernatant was discarded and the pellet washed with 1ml 70% (v/v) ethanol and then centrifuged for 5 minutes at 14000rpm. The pellet was dried using vacuum desiccator after ethanol was discarded. The dried pellet was dissolved in 100μl TE buffer, incubated for 5 minutes at 55 ^{o}C and stored at -20^{o}C.

2.4.2. Extraction of genomic DNA from plant tissues

Extraction of genomic DNA was performed using the DNeasy Plant Mini Kit (Qiagen, UK) according to manufacturer's instructions. 50 - 100 mg of desired frozen tissues were ground to a fine powder in liquid nitrogen with 400 microliter of AP1 (lysis buffer). The mixtures were placed in -80 ^{o}C freezer for 10 minutes, then mixture thaw out and add 4µl RNaseA. The mixture was incubated at 65 C for 10 minutes the lysate was transferred onto a QIAshredder spin column and centrifuged for 2 minutes at 16,000 g. The flow-through was transferred to a new microfuge tube containing 500µl of 100% ethanol. The mixture was then applied onto an RNeasy mini column and centrifuged for 15 seconds at 9,000 g. The flow-through was discarded and 500µl of Wash buffer 1 was added to the column before centrifuging for 15 seconds at 14,000 rpm. The DNeasy column was transferred to a new collection tube and 500µl of Wash buffer 2 was added and centrifuged for 2 minutes at 14,000 rpm. The flow-through was discarded and the column centrifuged for another minute to dry off the membrane and remove residual ethanol from the RPE buffer. The column was placed into a clean 1.5 ml microfuge tube and the RNA was eluted by adding 50µl Elution solution, allow to stand for 1 minute before centrifuging for 1 minute at 14,000 rpm.

DNA samples were quantified by spectrophotometer and DNA quality verified by gel electrophoresis before storage at -20 C.

2.4.3. Extraction of total RNA from plant tissues

Isolation of total RNA was performed using the RNeasy Plant Spectrum Mini Kit (Sigma-Aldrich, UK) according to manufacturer's instructions. 50 - 100 mg of frozen tissues were ground to a fine powder in liquid nitrogen with 500µl of lysis buffer containing 1µl of 2-Beta-mercaptoethanol. The mixture was left to thaw out and ground further to a homogenous lysate. The lysate was transferred onto a filtration spin column and centrifuged for 1 minute at 14,000 rpm. The flow-through was transferred to a new microfuge tube containing 500µl of binding solution. The mixture was then applied onto an RNeasy binding column and centrifuged for 1 minute at 14000 rpm. The flow-through was discarded and 500µl of wash buffer 1 was added to the column before

centrifuging for 1 minute at 14,000 rpm. The RNeasy column was transferred to a new collection tube and 500µl of wash buffer 2 was added and centrifuged for 2 minutes at 14,000 rpm. The flow-through was discarded and the column centrifuged for another minute to dry off the membrane and remove residual ethanol from the wash buffer 2. The column was placed into a clean 1.5 ml microfuge tube and the RNA was eluted by adding 50µl elution solution, allow to stand for 1 minute before centrifuging for 1 minute at 14000 rpm. RNA samples were quantified by spectrophotometer and RNA quality verified by gel electrophoresis before storage at -20 C.

2.4.4. Extraction of the plasmid DNA from bacteria

The plasmid DNA that has been transformed and isolated from the *E. coli* and the *A. tumefaciens* cells were put into isolation with the use of a GenElute^{TM} Plasmid Miniprep Kit (Sigma-Aldrich), and subsequently operated in accordance with the requirements specified by the, manufacturer. The culture was then developed over the period of an overnight from an isolated colony within the 5ml LB medium including the relevant antibiotics. Furthermore, a 1.5ml portion of the culture left overnight was then put into a microfuge tube and, for a period of a minute, centrifuged at 13000rpm per minute. This procedure was undertaken twice using the bacterial culture of the *E. coli* and four times for the culture of the *A. tumefaciens*; so that high yields of the plasmid DNA could be obtained. After this the pellet-cells were suspended once again in 250µl of resuspension buffer (containing RNaseA) and the supernatant thrown away.

The lysis buffer in the amount of 200µl was then used, it contained a dye of blue lysate, and this was added to lyse the cells and the container carefully inverted in order to make the mixture mix well. After this, 350µl of the Neutralization buffer was then placed into the mixture and the suspension was carefully mixed so that the cell refuse and the chromosomal DNA from the bacteria were blended. The lysed cells were then put in a centrifuge for a period of ten minutes a 13,000rpm, and the remaining supernatant was placed in a preparation spin column. This column was subsequently placed in a centrifuge for a period of an one-minute at the same rpm as before. A volume of 0.5ml of the wash solution-I was included into the mixture and placed into a centrifuge at a 13,000rpm. The container was cleaned with the use of 750µl of the Wash solution-II

that was mixed with 70% (v/v) ethanol and placed in the centrifuge at the same rpm for an additional minute. The column was subsequently moved to a 1.5ml centrifugal tube and 50µl of the elution buffer was mixed with the mixture at the center of the column. The column then remained standing for an additional minute before the mixture was one again placed in a centrifuge at 13,000rpm for an additional minute for the purpose of eluting the plasmid DNA.

2.4.5. Quantification of nucleic acids

Following RNA or DNA isolation, 1µl of the total RNA or genomic DNA was separated on a 1.5% agarose gel to evaluate the quality of the isolation. To measure the concentration of the nucleic acids, the absorbance at 260nm (Abs260) was measured using an Ultrospec4300 pro UV/Visible spectrophotometer (Amersham Biosciences). 1 Abs260 unit equates to 40 µg/ml of RNA or 50 µg/ml of double stranded DNA. Concentration of RNA in 1ng/ 1µl was calculated by multiplying 40, Abs260, and dilution factor. Also, concentration of DNA in 1ng/ 1µl was calculated by multiplying 50, Abs260, and dilution factor.

2.5. Amplification of DNA by Polymerase Chain Reaction (PCR)

2.5.1. Design and storage of the oligonucleotide primer

Various primer sets of the oligonucleotide primer were intended to amplify the genomic DNA and cDNA, as well as the cloned products and the sequence of vectors. The primers were designed with the intention of being utilized manually or else with the use of the MacVector. In the case of the genomic DNA, and the cDNA sequential amplification procedure, various primers were developed of an 18-25bp length and a T_m (melting temperature) of 45-65^oC. Whenever it was possible, a GC clamp was used at the three-inch end of the primer. In the case of the Gateway recombination stage, the primers were inclusive of the relevant 12bp, upon locations that were deemed necessary for the recombination. Each and every one of the primers were sourced from Sigma-

Aldrich and, using a suitable amount of Tris buffer, resuspended in the 10mM Tris-Cl of pH value 8.0. This was done in order to realize a concentration of the stock at 0.1ml. A working solution of primers of a volume of 5μM was then prepared for use in PCR reactions, and all the primers used kept at a temperature of 20^{o}C.

2.5.2. Conditions for the PCR

The conditions that were employed in the PCR stage were differential regarding the specific size of the product they were to amplify, in accordance with the T_m of each of the forms of product to be used and primers to be used. DNA was denatured at 96^{o}C within BioTaq enzyme, or else 98^{o}C in the Velocity phusion polymerase. The temperature annealing of the T_a was established to be lower than the T_m and thus was needed for the best annulation temperature, which was established though the use of gradient PCR. The extension time was established as 72^{o}C for a period of thirty seconds for each kb of the product itself using the BioTaq and fifteen seconds for each kb of the product in the case of Velocity phusion polymerase. A lower-limit for the number of cycles was established in the case of amplifying the promoters as well as the sequential coding required for the cloning procedure. The PCR reactions were undertaken using one of three thermo-cycles; the TProfessional Basic Gradient (sourced from Biometra) the Primus 96 Plus (sourced from MWG-Biotech) and the Mastercycler (sourced from Eppendorf) the precise parameters of the BioTaq and the Velocity phusion PCR.

Reagents	Volume	Final concentration
Deionized water	11.4 µl	
10 X NH_4 buffer	2.0 µl	1X
50 mM $MgCl_2$	1.0 µl	2.5mM
10 mM dNTPs	0.5 µl	0.25mM
5µM Forward primer	2.0 µl	0.125µM
5µM Reverse primer	2.0 µl	0.125µM
5.0 U/µl Taq polymerase	0.1 µl	
DNA template	1.0 µl	
Total Volume of reaction	20.0µl	

2.5.3. General PCR

The reagents of PCR, including $MgCl_2$, the 10xNH_4 buffer, as well as the BioTaq polymerase, were all sourced from Bioline. The required dNTPs were sourced from wither one of Invitrogen or Bioline from the UK. To amplify the cloning products, the proofread Velocity Phusion DNA polymerase was employed as replacements. The DNA template was diluted to 1 to 100-part mix within a plasmid for the genomic DNA, and 1 to 10 parts for the cDNA. The factors that employed to amplify the PCR products that changed depending on the product size, melting temperature, and primer. Annealing temperature was set up five to ten degrees Celsius lower than the primer's melting temperature, and gradient PCR was utilized to determine the best annealing temperature. The entire DNA was denatured, either at 96^{o}C (BioTaq) or 96^{o}C (Velocity). The extension time for the primer was put at 72^{o}C for a period of half a minute for each kb of the substance in the case of BioTaq or else 15 seconds for each kb

with Velocity Phusion. A lower-limit of the cycles that must be used in the procedure of amplifying promoters and the coding sequences that are used in the cloning procedure. The PCR reactions were performed with the intention of using one of three different thermocyclers.

In order to undertake a general PCR reaction with the use of BioTaq DNA (polymerase) along the limitations used, these were: Denaturation for twenty seconds at 96^{o}C; annealing for half a minute at a temperature of 55-65^{o}C; and extension for half a minute-per-one kb at a temperature of 72^{o}C and lastly 72^{o}C for a period of five minutes.

2.5.4. High fidelity PCR for cloning

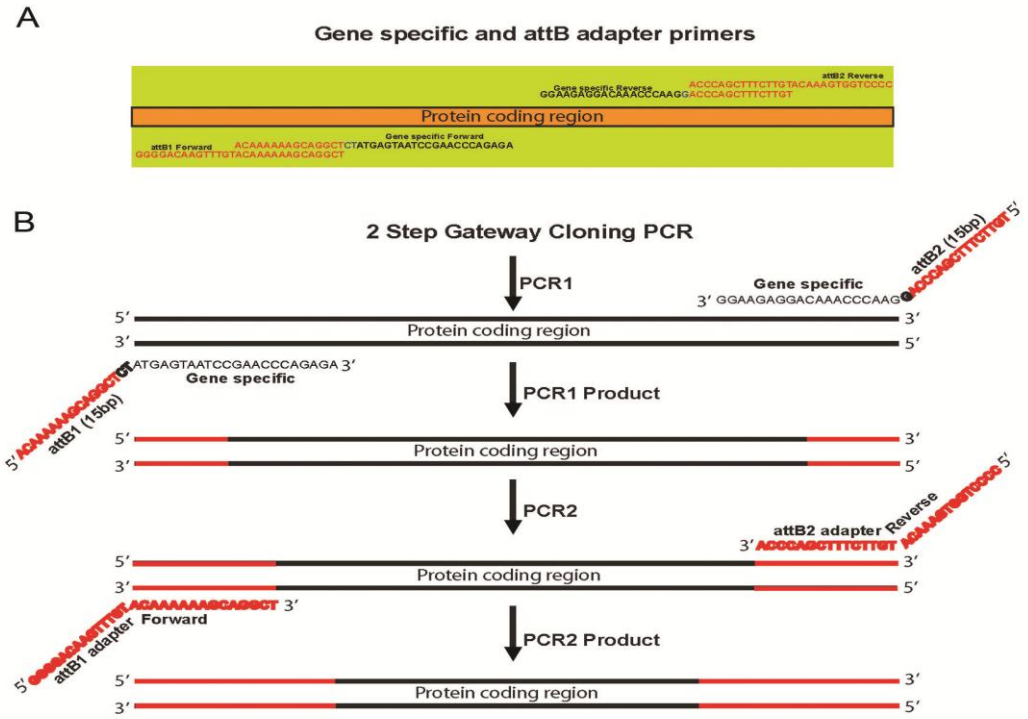


Figure 2.1: 2-steps Gateway cloning PCR reaction. In the case of recombinational cloning (Gateway®), the PCR materials were amplify through the use of Velocity Phusion® High-Fidelity Polymerase, which creates PCR products using high efficiency and close amplification. The specific primers of the genes held a 15 base pair that overlapped with the attB primers so that designation of costly and extensive primers could be avoided. The initial PCR1 reaction was undertaken to create the needed products and the second PCR reaction was undertaken using the relevant attB adapted primer utilizing the amplify products used in the PCR1 experiment so to expand the attB sites before the recombination Gateway® procedure. The initial PCR includes an element of the attB that was recombination procedure within the PCR-1 product, converse to the second PCR that advances using reversed and forwarded primers that create the complete attB1 and attB2 sites, creating the substrate attB-PCR, which can be used in the recombination process. The required buffer that employed so to create master-mixes, and thus given the Phusion-enzyme. The dNTPs were also given Invitrogen or Bioline, and the DNA diluted to a single part per-hundred for the plasmid-DNA and genomic-DNA for PCR1. (Adapted from (Nadia Taimur, 2012))

Phusion PCR reaction for PCR1

Reagents	Volume	Final concentration
Deionized water	14.2 µl	
5X HF buffer	5.0 µl	1X
5mM $MgCl_2$	1.0 µl	0.2mM
10mM dNTPs	0.5 µl	0.2mM
5µM Forward primer	1.5 µl	0.1µM
5µM Reverse primer	1.5 µl	0.1µM
Phusion polymerase (2U/µl)	0.3 µl	0.6U
DNA template	1.0 µl	
Total Volume of reaction	25.0 µl	

Phusion PCR reaction for PCR 2

Reagents	Volume	Final concentration
Deionized water	15.4 µl	
5X HF buffer	10.0 µl	1X
5mM $MgCl_2$	2.0 µl	0.2mM
10 mM dNTPs	1.0 µl	0.2mM
5µM attB adapter Forward primer	8.0 µl	0.8µM
5µM attB adapter Reverse primer	8.0 µl	0.8µM
Phusion polymerase (2U/µl)	0.6 µl	0.6U
DNA template (PCR-1 product)	5.0 µl	
Total Volume of reaction	50.0 µl	

2.6. Purification of DNA

2.6.1. Purification from agarose gels

DNA fragments were excised from agarose gels for sequencing or cloning using the GenElute^{TM} Gel Extraction Kit (Sigma-Aldrich). The gel was weighed and three gel volumes (100mg=100 µl) of Gel Solubilization Solution were added. After that, the mixture was incubated at 55 ^{o}C for 10 minutes. One volume isopropanol was added when gel was completely dissolved in the mixture. The sample was loaded into binding column and centrifuge for 1 minute at 13200rpm. The flow-through was discarded and 700µl wash solution was added to the column and centrifuge for 1 minute at 13200 rpm. After discarded the flow-through centrifuged again for 1 minute without any additional wash solution to remove excess ethanol. The binding column was replaced into a new collection tube 50µl elution buffer was added to the middle of the column and incubated at room temperature for one minute, and then the DNA was eluted by centrifugation at 13200 rpm for 1 minute. DNA was used directly for cloning reactions or stored -20^{o}C.

2.6.2. PCR product purification

GenElute^{TM} PCR cleanup (Sigma-Aldrich) kit was used to get higher recovery of purified DNA. 5 volumes of Binding Solution were added to 1 volume of the PCR product and vortexed. The solution was transferred into the binding column and then centrifuged at 13200 rpm for 1 minute. The binding column was replaced into the collection tube. 500µl Wash Solution was applied to the column and then centrifuged at 13200 rpm for 1 minute. The column was centrifuged at 13200 rpm for 2 minutes, without any additional wash solution, to remove excess ethanol. The column was transferred to a sterilized new 2 ml microfuge tube. 50 mL Elution Solution was applied to the middle of the column and then incubated at room temperature for 1 minute. The DNA was eluted and centrifuged at maximum speed for 1 minute. The PCR product is ready for immediate use or storage at -20^{o}C.

2.7. First-strand cDNA synthesis by Reverse-Transcription PCR (RT-PCR)

2.7.1. DNAse Treatment of total RNA

The total RNA isolated from plant tissues was first DNAse treated with Amplification Grade DNase I (Sigma-Aldrich) to remove any DNA contamination. Depending on the volume of total RNA, the 10x Reaction Buffer was diluted to 1x and the same volume of DNaseI added to the sample followed by incubation at room temperature for 15 minutes. The same volume of STOP solution was then added and the sample incubated in the thermocycler during at 70^{o}C for 10 minutes to denature the DNaseI. Samples were kept on ice in preparation for first-stand cDNA synthesis.

2.7.2. Reverse transcription reaction for RT-PCR

First strand cDNA was produced with total RNA primed with oligodT primer using M-MLV reverse transcriptase (Promega). 1000 ng/µl of total RNA was used to get first strand cDNA. Also, Reverse transcription negative control reaction that contains no M-MLV was included during cDNA synthesis. The first strand cDNA was serial diluted (1:5, 1:10, 1:20) to use as a template for BioTaq PCR reactions using specific primer pairs. The mixture was briefly vortexed and put in a heated thermocycler at 42^{o}C for 1 hour. The cDNA was used directly for PCR reaction as a template or stored -20^{o}C. The master mix of the cDNA reaction recipe as follows;

Reagents	Plus (+) RTase	Minus (-) RTase
Water (RNase free)	up to 25 µl	up to 25 µl
5X Reaction buffer (M-MLV)	5.0 µl	5.0 µl
RNase inhibitor	1.0 µl	1.0 µl
dNTPs Mix	1.25 µl	1.25 µl
OligodT	1.0 µl	1.0 µl
Total RNA	1000 ng/µl	1000 ng/µl
M-MLV RT	1.0 µl	-
Total Volume of reaction	25.0 µl	25.0 µl

2.8. DNA modification, cloning and sequencing

2.8.1. Digestion of DNA

PCR product and plasmid DNA were carried out with different enzymes to restriction digest according to manufacturer instructions. The reaction was performed in a proper buffer and incubated at 37^{o}C for approximately two hours. The restriction digest reaction was established as follows;

Restriction digest recipe	Volume
H_2O	Up to 10µl
Cut smart buffer	1.0 µl
10X BSA (1mg/ml)	1.0µl
PCR product or DNA	5.0 µl or 1 µg
Restriction enzyme (4U/ µl)	0.1 µl
Total Volume	10 µl

All restriction enzymes and buffers were bought from New England Biolabs.

2.8.2. Sequencing of DNA

Plasmid DNA was sent to Source BioScience (Nottingham, UK) for comparison predicted and clone sequence. 100ng of plasmid DNA in a total volume of 10µl was sent based on company requirement. For entry clones, M13 primer pairs were used to get sequence recombined inserts. Forward and reverse primers that based on the length of the sequence were sent to cover full sequence. Fasta format of the sequence was downloaded from the website (http://www.lifesciences.sourcebioscience.com/) ,and analysed with the MacVector software. 'Align to reference', which is the feature of the MacVector, was used to verify predicted and clone sequence.

2.8.3. Cloning by Gateway® recombination

The Gateway® Cloning System (Invitrogen, UK) was used for cloning. In this recombinational cloning process, DNA fragments were flanked by PCR using primer pairs, which contain recombination sites (attB1 and attB2 sites). The Gateway® system carries out two reactions, to clone PCR products into expression vectors.

1- BP reaction

To generate entry clones in a BP reaction 125ng PCR product and 75ng of donor vector were used. The mixture was incubated at room temperature for overnight, and then the reaction was terminated by addition of 0.5µl of proteinase K and incubated at 37^{o}C for 5 minutes.2.5 µl of the total volume was used for transformation into *E.coli* cells (A-select) of 10^{8} efficiency (Bioline). Colony PCR was performed using M13 primer pair to screen the colonies. To set up 5ml overnight cultures positive colonies were used for isolation of plasmid DNA from the cultures. Isolated plasmid DNA was sent for sequencing to verify before use in LR reaction.

BP reaction	**Volume**
PCR product	125ng
PDONR vector	75ng
BP Clonase- II plus	1.0µl
TE buffer	Up to 5.0µl
Total Volume	5.0 µl

2- LR reaction

The entry clones that had been verified were used to recombine the insert into the destination vector within the LR-reaction. In the case of a single-site LR reaction, the recombination of the 125ng that can be seen in the entry clone as well as the 75ng of the desired for destination vector were employed in the reaction of the 2nd ligation; catalyzed with the use of the LR Clonase II-enzyme from Invitrogen. In the case of the Multisite-LR reaction, the recombination of the 5fmol of all of the entry clones and the 10fmol of the required destination vector were thus catalyzed alongside the LR enzyme of Clonase II Plus from Invitrogen. This experiment too was left in an incubator overnight at ambient temperature before being terminated with the use of proteinase K enzyme (of 0.5µl volume) and set at a 37^{o}C temperature or a period of 10 minutes,

while 2.5µl of the reaction in total was used to transform the *E. coli* cells of the α-select, and then the colonies were determined as valid or not by the use of the PCR. Those colonies that were screened to be positive, were inoculated into the 5ml of the LB culture overnight, and the recombinant plasmid was separated and used for the purpose of restriction assessment before the sequencing procedure could progress The verified vectors of expression were consequently transform into *A. tumefaciens* (GV3101) for the alteration into *Arabidopsis*. Single and multi sites LR reaction recipe as follows;

Single-site LR reaction	**Volume**
Destination vector	75ng
pDONR221 entry clone	125ng
LR Clonase II	1.0µl
TE buffer	to 5.0µl

Two-site LR reaction	**Volume**
Destination vector	10fmol
pDONRP4-P1R entry clone	5fmol
pDONR221 entry clone	5fmol
LR Clonase II Plus	1.0µl
TE buffer	to 5.0µl

Three-site LR reaction	Volume
Destination vector	10fmol
pDONRP4-P1R entry clone	5fmol
pDONR221 entry clone	5fmol
pDONRP2R-P3 entry clone	5fmol
LR Clonase II Plus	1.0µl
TE buffer	up to 5.0µl

The formula to calculate the ng of plasmid DNA needed to achieve the desired f mols is as follows:

ng needed = desired f mol x size of vector (bp) x (660 x 10-6)

2.9. Gel electrophoresis

2.9.1. Agarose gel Electrophoresis for DNA separation

According to (Sambrook *et al.*, 1989), 1.5 g of agarose is melted in 100 ml of 1 x TAE buffer to make 1.5 % gel. The molten gel was cooled to 65 ^{o}C and 2µl ethidium bromide was added to the gel. It mixed thoroughly and then poured into a gel tray with comb. The gel was allowed to set for 20 minutes at room temperature, the comb was carefully remove and the solidified gel was place into an electrophoresis tank containing 1 x TAE buffer. DNA samples were loaded into the wells after mixing it with gel loading dye (Orange G powder dissolved in 50% glycerol).

Gel was run for approximately 30 minutes using a voltage of 8V/cm. The DNA fragments were visualized using an UVP trans-illuminator and a thermal gel print was taken with a graphic printer (UP-895CE, Sony). The DNA fragments size was determined by using standard DNA ladder (NEB).

2.10. Generation of transgenic plants

2.10.1. In planta transformation of *Arabidopsis thaliana*

The plants of the *Arabidopsis* were transformed through the 'floral' dip methodology (Clough & Bent, 1998). The plants that were supposed to be healthy were developed in a continuous light schedule, with their first bolts clipped when they reached a 2-5cm in height. Plants that grew auxiliary buds for around a week were subsequently completely developed with regard to their inflorescences that were trimmed and the plants given Miracle-Gro All Purpose Concentrated Plant Food (sourced from Scotts). After a period of a one-week, the plants were subsequently prepped for transformation through the clipping of their siliques and completely opened flowers. A single colony of the *Agrobacterium* was inoculated in the 5ml culture of the LB that was containing relevant antibiotic choice. *Agrobacterium* cells that permitted to develop overnight in the 28^{o}C shaker, with the use of intense shaking at 220 rpm. An aliquot of the culture was then subcultures into a single 1-litre flask that contains 400ml of fresh LB medium, along with the selection of antibiotics and the saturation was cultivated over the period of an overnight at a temperature of 28^{o}C with the use of intense shaking at 220rpm. Subsequently, the cells were then further centrifuged at 4600 revolutions per minute for a period of twenty minutes and then re-suspended in a medium of a single litre of infiltration; 10 micrograms per litre of 6-benzylaminopurine; 2.17 grams per litre of half strength MS salt, 3.16 grams per litre of full-strength Gamborg B5 vitamins; 3.16 grams per litre of full-strength Gamborg B5 vitamins; 0.5 grams per litre of MES, 50 g/l sucrose. After this, 300µl of Silwet L-77 was combined with the re-suspended culture (sourced from Vac-In-Stuff and Lehle Seeds). Finally the above ground area of the plants was dipped in the solution of the Agrobacterium with the use of mild agitation for a period of around forty-five seconds. Plants that had been doused in the solution were then retained beneath a nylon autoclave bag for 36-48 hours within a high-humidity atmosphere. The plants were then kept and developed ordinary in accordance with normal watering and care, their stems and branches staked together with tie twists. After the siliques had matured, the watering was stopped and the organisms retained within a drying room for a period of around a two weeks. The dried-seeds were harvested and placed on plated and/or solid that contained the correct choice with which

to identify the primary, relevant transformations.

2.10.2. Transient transformation of *Nicotiana tabacum* leaf

Following the methodological protocol of agrobacterium infiltration, as suggested by (Sparkes *et al.*, 2006) with the use of the Sigma reagents, transient change from the *Nicotiana tabacum* plant leaf of the Agrobacterium that was mediated with short-lived transformation of *Nicotiana tabacum* leaf within *Nicotiana tabacum cv. SR-1* epidermal cells from the leaf. Isolated colonies of the changed Agrobacterium tumefaciens GV3101 (pMP90) were inoculated into 5ml of the LB medium that held the relevant antibiotics and this was subsequently held overnight and incubated at $28^{o}C$, along with continuous shaking of 200rpm. The plant cells were then taken from a 1.5ml sample of the held substance though the use of a centrifuge, and the pellet extracted re-submerged in a 1ml of the infiltration medium—0.1 mM acetosyringone; 50 mM MES; 2mM $Na_3PO_4.12H_2O$ and 280mM D-glucose. Cells were then washed through their re-suspension in another 1ml of the infiltration medium in order to get rid of any traces of the antibiotic and the OD600 with the use of Pye Unicam PU 8650-spetrophotometer (sourced from Phillips of the Netherlands). All of the agrobacterium solutions were subsequently inoculated in accordance with the methodology of the experiment at an OD600 of 0.1 for the reporter vector and the effectors vector and at strength of OD600 of 0.02 for the Renilla luciferase vector (the control vector). The four to six week-old *Nicotiana tabacum* plants were then developed in greenhouse conditions though also were placed under a white fluorescent light for a period of one hour before their infiltration to guarantee that their stomata were fully opened.

The 3^{rd} and 4^{th} sizeable leaves, from the apical meristem of the plant were, generally speaking, selected for the process of infiltration. Each infiltration stages were undertaken three times using both of the sides of the midrib region of a couple of distinct leaves. The solutions of the *Agrobacterium* were sucked into 1ml syringes and the lower side of the leaf prepped by pressing a $0.5cm^2$ area with the solution to get rid of the cuticle. The end of the syringe was then placed to the rubbed regions than the agrobacterium in the solutions carefully infiltrated into the plant with the aid of pressure by the technician's finger.

The areas that had been infiltrated were demarked and denoted with a black pen with permanent ink. The gloves used were covered in spray of 70 percent (v/v) IMS between each of the leaves to stop contaminative elements. The areas that had been infiltrated were subsequently excursed in dual luciferase assays. The leaves and their plants were then placed in a growing chamber (which kept ordinary conditions) and sat for two days.

2.10.3. Dual luciferase assays

The Luciferase reporter activity that comes from the transient expression of assays was determined following an altered protocol of the dual luciferase report assays, in accordance with (Sherf *et al.,* 1996). Agrobacterium-infiltrated *Nicotina tabacum* leaf areas were utilized through a size seven-cork borer. The leaves that had been excised were put into a pestle and mortar for the process of grinding with a 0.5ml of 1x Passive Lysis Buffer (Promega) unto the point at which extracts seemed to be homogenous (in roughly thirty seconds). The extracts taken from the leaf were put into a centrifuge for 14.000g at a temperature of 4^{o}C for the collection of supernatant from the leaves. This residue was then put on ice while the dual luciferase action was undertaken. Enzyme buffers of fresh luciferase were determined and substrates assimilated following the injection of the Clinilumat LB9502 luminometer (Berthold). The whole of 20ml of the firefly luciferase assay buffer in addition to 20ml of Renilla luciferase were employed to be sufficient for each of the injectors to be primed for the assaying of different leaf extracts. Several Luciferase reactive buffers were prepped with a small alteration of the published non-commercialized recipes, according to (Dyer *et al.,* 2000). The initial firefly luciferase assay buffer; 25mM glycylglycine; 0.1mM CoA, 75µM luciferin with final pH adjusted to 8.0); 15mM KPO_4 (of pH 8.0); 4mM EGTA, 2mM ATP, 1m M DTT, 15mM $MgSO_4$.

Furthermore, a Renilla luciferase assay buffer of; 0.22 M KPO_4 pH 5.1, 0.44mg/ml BSA 2.2mM Na_2EDTA, 1.43µM coelenterazine with final pH adjusted to 5.0; and 1.1 M NaCl were also prepped. Injectors of the luminometer were subsequently set with assay buffers in addition to two distinct 25µl aliquots, which were separately assayed for the purpose of luciferase action with 200µl of each of the buffers. The luciferase

action was a process of counting photons that were taken at 10-second intervals with an integration time of one second. The un-infiltrated leaf of the tobacco was then assayed to determine the background of the leaf and this was taken from the luciferase activity of regions that had been infiltrated through numerical subtraction. A normal, or 'normalized' dual of the luciferase actions (Fluc/Rluc) was determined for each of the infiltrations through the process of divided the firefly luminescence action (Fluc) with that of the luminescence activity of Rluc.

2.11. Cytological analysis

2.11.1. DAPI staining of pollen nuclei

The visualization of the pollen nuclear morphological process was undertaken with the dying of DAPI (4‘, 6-diamidino-2 phenylindole), in accordance with recommendations made by former literature (Park et al., 1998). Four to five completely opened flowers were placed into an eppendorf tube that held 0.3ml of 10µg/ml DAPI in a GUS buffer (0.1M sodium phosphate pH 7.0mM EDTA—0.1 percent (v/v) Triton x-100. Dissolving powered DAPI into water at 0.4mg/ml DAPI with high-grade sigma created the DAPI. The grains of pollen that were released into the DAPI solution were done so though vortexing, after which the solution was centrifuged for five seconds in a picofuge (sourced from, Stratagene in the UK). In total, 3µl of the pelleted pollen was transferred to a microscope's slide, which gently had pressure exerted upon it and fastened shut with nail polish to ensure that the sample would not dry out. This was then seen under a fluorescent ECLIPSE 80i microscope (sourced from Nikon Japan) or alternatively a confocal scanner laser microscope ECLIPSE TE2000-E (also sourced from Nikon Japan).

For the purposes of screening the pollen from a huge sample group, the mature pollen which had been taken from one or two single opened flowers were placed into a 96-well microtiter plate that held 0.1ml of DAPI solution. The individual grains were released from the flowers with a light tap upon the microtiter slate and the nuclei of the grains were seen under an inverted fluorescent microscope (sourced from Zeiss Axiophot 100).

2.11.2. Fixation of anther tissue of Magnolia species

To fix anthers from different *Magnolia* species, freshly prepared fixative was used. The fixative was made by adding 1 part of glacial acetic acid and 3 parts of ethanol (95 to 100%). Anthers were fixed for 24 hours and then transferred to an eppendorf tube containing 300 µl of 0.8 µg/ml DAPI staining solution (0.1 M sodium phosphate, pH 7; 1mM EDTA, 0.1% (v/v) Triton X-100, 0.5 mg/ml DAPI; high grade, Sigma).

Fixative solution

Reagents	Volume
Glacial acetic acid	1 part
Ethanol (95% to 100%)	3 parts

2.12. Microscopy and image processing

Each of the images was taken using a various cameras and objectives that depending on the microscopic equipment used and the particular image that need to be taken. In using an ECLIPSE TE2000-E (sourced from Nikon of Japan) an inverted image of fluorescent microscope along with a mercury microscope as a source to excitement alongside plan Flour 40x/1.3Na oil, or a Plain flour 60x/1.25 NA oil immersion objectives were used. Several DIC images were taken using a micropublisher 3.3 RTV CDD color camera (QI-imaging, Canada) and the fluorescence images were taken using an ORCA-ER CCD camera (cooled) taken from Hamamatsu Photonics, in Japan (model number C4742-95-12ERG). The images that were taken were viewed in advance, taken and stored using NIS-Elements Basic Research v3.0 software (Nikon, Japan). In using the ECLIPSE 80i camera (from Nikon in Japan) and light emitting diode excitation source was also employed in the form of a CoolLED from presicExcite, along with the use of a Apo VC 60x/1.4 or a Plan Flour 40x/1.3 Na oil immersion objective. The fluorescence images were taken with the use of a cooled-CCD camera (a DS-QiMc from Japan) and were stored and processes with the use of NIS-elements software (basic elements v3.0

of Nikon Japan) in the format of Jpeg-2000 image format. With regard to the CLSM (con-focal laser scanning microscopy) the use of the confocal module (from Nikon of Japan) was used alongside a Nikon camera (ECLIPSE TE2000-E model form Nikon Japan).

For the laser-excitation sources two devices were used; the Melles Griot Argon Ion (at an excitation level of 488 nm) and the Melles Gruit Melium-Neon camera (at an excitation level of 543nm). The CLSM was utilized through the use of a EZ-C1 controlling divide and the relevant image-capture computer program. Images taken of the dissected siliques were taken with the employment of a 3- video CCD color camera; model number KY-F55B form manufacturer JVC, which was mounted on a Zeiss dissection microscope (model number STEMI SV8). The camera was attached to an imaging capture card (a Neotech IGPCI) and the files shown on the software programme; Image Grabber PCI 1.1. The computer used for this process ran a Mac OS9 operating system and all images that were processed were done so with the use of version CS5.1 of Photoshop.

2.13. Statistical analysis

Statistical analysis of the data's was tested using Microsoft Excel and statistical software package GraphPad Prism 6.0. Statistical analysis of the transactivation of three part constructs was undertaken with the help of two-paired T-test that find out two values in same population. Statistical analysis of the percentage of tricellular pollen was undertaken with the help of two-way ANOVA (analysis of variance) test, which evaluated differences in the examined various temperature condition and time (day). In order to determine difference data sets in multiple comparisons Tukey's multiple comparison test used for each temperature treatment.. All tests were two-sided and a p-value less than $\alpha = 0.05$ was considered to be statistically significant. The statistical evaluation of germ cell division and male transmission was performed with the Chi-square ($\chi 2$) test using Excel in order to determine whether the ratio of observed phenotype was significantly different from the expected ratio of phenotypes. Statistically significant outcomes were determined using α level of 0.05.

Chapter 3

COEXISTENCE OF POLYMORPHIC POLLEN IN MAGNOLIACEAE

3. Coexistence of Polymorphic Pollen in Magnoliaceae

3.1. Summary

Background

In 1926, Schürhoff (1926) posited that the bicellular state was genetically responsible for the tricellular state with the earliest account of the different taxonomic distributions of each being composed by Schnarf in 1939. Three decades later, (Brewbaker, 1967) analyzed approximately 2,000 different angiosperm species and his findings substantiated the theory proposed by Schürhoff. Although Brewbaker reported all basal angiosperm families are exclusively bicellular, the presence of tricellular pollen was subsequently reported in some extant basal angiosperm species (i.e. *Annona cherimola*) from the Annonaceae (Rosell *et al.*, 1999) and *Liriodendron tulipifera* from Magnoliaceae (Johri *et al.*, 1992). Moreover, an environmental condition such as temperature was shown to affect the rate of pollen development and tricellularity in *A.cherimola* (Lora *et al.*, 2009).

Results

This study seeks to obtain data to readdress the report by Brewbaker who noted that Magnoliaceae produce exclusively bicellular pollen. Forty-one Magnolia species have been received from UK, USA and China to observe pollen cell number. Twenty-seven Magnolia species showed the existence of some tricellular pollen grains and fourteen Magnolia species produced exclusively bicellular pollen. The highest percentage of tricellular pollen, which was approximately 20%, was observed in *Magnolia grandiflora* and *Liriodendron tulipifera.* The percentage of tricellular pollen was found to increase during development until anther dehiscence and the highest proportion of tricellular pollen was observed when pollen matured under higher temperature conditions.

Conclusion

The major finding is that 68% of Magnoliaceae species analysed showed polymorphic (bicellular and tricellular) pollen instead of exclusively bicellular pollen. Maturation of pollen under increased temperature regimes can significantly accelerate division of the generative cell in *M. grandiflora*, which suggests that temperature as well as genetic factors may contribute to the observed variation in pollen cell number polymorphism observed.

3.2. Introduction

The duration of the sperm cell division stage varies according to species. In 70% of angiosperms (Brewbaker, 1967) and all nonflowering seed plants (Friedman, 1999; Rudall & Bateman, 2007), bicellular pollen is released from the anthers of angiosperms as pollen mitosis II occurs following pollen germination. Nonetheless, pollen mitosis II also takes place prior to germination in various families and genera, which means that tricellular pollen is released. According to Brewbaker (1967), the reasons behind this remain unknown though tricellular pollen display common attributes in that they often germinate quickly yet have a short lifespan.

Schürhoff (1926) proposed that the bicellular state was genetically responsible for the tricellular state with the earliest account of the different taxonomic distributions of each being composed by Schnarf in 1939. Three decades later, Brewbaker (1967) analyzed approximately 2,000 different angiosperm species and his findings substantiated the theory proposed by Schürhoff. However, this study also discovered that species belonging to the same group, family or order typically possessed a set number of pollen nuclei. Thus, according to Webster et al. (1982) and Johri et al. (1992), though Schürhoff's theory was not discounted, several revisions were required. For instance, Webster *et al*. (1982) and (Soltis *et al.*, 2005) assert that tricellular pollen developed independently in many cases with no indications that a reverse transition ever took place. The evolution from bicellular to tricellular is a biological process that has been identified in many conditions, with many attributing this development to the need for more rapid germination (Brewbaker, 1967; Bergamini Mulcahy & Mulcahy, 1988).

Within the eumagnoliid clade, Brewbaker (1967) states that all basal angiosperm families release bicellular pollen, including the Magnoliales, Laurales, Piperales, and Canellales orders (APG II, 2003; Soltis *et al*., 2005). A minimal number of tricellular-producing plants have been identified aside from *Liriodendron tulipifera* (Magnoliaceae) and certain species that form part of the Calycanthaceae and Monimiaceae families (Johri *et al*., 1992). As a result, taxonomists were surprised to discover that *Annona cherimola* of the Annonaceae family and the Magnoliales order produced tricellular pollen.

There have been few cases of both tricellular and bicellular pollen being released from the anther yet Maheshwari (1950) mentions some instances identified in a variety of plants. Similarly, (Sampson, 1969) and (Gardner, 1974) discovered both types of pollen within the Laurales order (Magnoliidae), Johri *et al*. (1992) discovered that both types existed in the Olacaceae (order Santalales) and Webster and Rupert (1973) identified cases in the Euphorbiaceae (order Malpighiales). Grayum (1985) also observed similar characteristics within the Araceae family, which is part of the Alismatales order; this order is also included in the lineage of monocots (Soltis *et al.*, 2005). These cases are often interpreted as biological anomalies and have not been studied in any great depth. Nonetheless, these instances could indicate a phase of transition or development between a bicellular and tricellular condition. Several factors, both internal and external, determine the number of nuclei present in pollen. In particular, temperature is known to impact upon reproductive activities (Prasad *et al.*, 2000; Sato *et al.*, 2002) and the duration of meiosis (Bennett, 1977). In addition, temperature affects the lifespan and performance of pollen (Delph *et al.*, 1997; Hedhly *et al.*, 2005) as well as the duration of the mitosis stage (Klindworth & Williams, 2001). In an attempt to determine whether or not the temperature to which pollen is exposed during development has any impact on the number of nuclei present, the nuclei of *A. cherimola* pollen grains were counted during a development sequence at varying temperature levels (Lora *et al*., 2009).

The major aim of this study was to assess the validity of Brewbaker's evidence that Magnoliaceae species possess exclusively bicellular pollen at anthesis. This was investigated by analysing pollen from herbarium and living specimens of forty-one *Magnolia* species. These were sourced from the Natural History Museum herbarium and various botanical gardens from United Kingdom, United States, and China. A

second aim was to investigate whether temperature affects pollen development and the frequency of formation of tricellular pollen at anthesis in *Magnolia grandiflora*. In order to examine whether temperature could affect the rate of division of the generative cell the number of pollen nuclei was scored in flowering branches of *M.grandiflora* cultured under different temperature regimes in controlled growth chambers.

3.3. Results

3.3.1. Investigation of tricellular pollen type in *Magnolia grandiflora*

This project was initiated with an original aim to investigate whether the *DUO1* gene is conserved in early angiosperms pollen, especially *Magnolia grandiflora*. To identify the *DUO*1 gene in *M. grandiflora,* pollen was collected during flowering time from Beaumont hall, University of Leicester botanic garden. According to the early published literature, Magnoliaceae and all basal angiosperms produce exclusively bicellular pollen (Brewbaker, 1967). In order to confirm this finding *M.grandiflora* stamens were fixed and pollen was stained with DAPI. Surprisingly, Michael Borg observed *M. grandiflora* total pollen population have polymorphic pollen type instead of exclusively bicellular pollen type. This is the first demonstration that species of Magnoliaceae family have both bicellular and tricellular pollen type. This result stimulated the question: is polymorphic pollen only present in this *M. grandiflora* individual and flower? In order to answer this question pollen from different flowers collected from two different locations, which are Beaumont Hall and Belgrave Hall and different trees, was observed with epifluorescent microscopy and pollen nuclear number was scored for each flower. Analysis of pollen phenotype showed a polymorphic pollen with a large number of bicellular pollen and comparatively lower number of tricellular pollen clearly showing two sperm cells. Variations in the numbers of tricellular pollen were observed even in the same flower. The frequency of tricellular pollen analyzed from different stamens from the same flower varied from 3% to 15%. Overall 1% to 23.9% of tricellular pollen was observed in different flowers collected from different trees (Table 3.1).

Table 3.1: Pollen from different flowers of *Magnolia grandiflora* from Beaumont Hall and Belgrave Hall showing different frequencies of polymorphic pollen. *Magnolia grandiflora* flowers were collected from different trees at different locations. The pollen nuclei were observed with epifluorescent microscopy and counts were made for each flower. The number of pollen grains scored per sample was ≥ 100, Abbreviations: PM, polymorphic, TCP, tricellular pollen;, UOLBG; University of Leicester Botanic Garden).

Species Name	Flower	%TCP (n)	Pollen Type	Source
Magnolia grandiflora	A	1.4%	PM	Beaumont Hall (UOLBG)
	A	1.1%	PM	
	B	1.6%	PM	
	C	1.2%	PM	
	D	8.4%	PM	
	D	6.1%	PM	
	D	11.4%	PM	
	D	10.7%	PM	
	D	15.8%	PM	
	D	11.4%	PM	
	D	3.6%	PM	
Magnolia grandiflora	A	9.9%	PM	Belgrave Hall and gardens
	A	11.2%	PM	
	A	5.3%	PM	
	B	23.9%	PM	

The results obtained from the preliminary analysis of pollen germ cell number in *M. grandiflora* sources from Leicester stimulated the question of whether the phenomenon was restricted to local samples or extended to geographically separated individuals in the USA to explore the *M. grandiflora* pollen type in USA, all university botanic garden websites were examined especially southeast USA because *M. grandiflora* is endemic to that area. Bruce Kirchoff who is a Professor in the department of Biology at the University of North Carolina Greensboro, provided *M. grandiflora* from five different trees in the university campus. Dixie Z. Damre who is a curator in the Clemson University herbarium provided stamen samples from three different *M. grandiflora* trees from different locations on the campus. Michael Wenzel who is a plant recorder in the Atlanta botanical garden provided dehisced stamens from one *M. grandiflora* individual. Dr Lisa Wallace who is an associate professor and curator of the herbarium in Mississippi State University provided dehisced stamens from one *M. grandiflora* individual. Prof. David Twell provided dry dehisced stamens from one *M. grandiflora* individual from Porto.

All fresh stamen samples were fixed with 3:1 ethanol-acetic acid for 24 hours, then pollen was dissected from stamens and stained with DAPI solution. Overall, twelve *M. grandiflora* pollen samples were analysed and remarkably, ten out of twelve were found to show polymorphic pollen , between 1.5% to 21% tricellular polen. Only, two *M. grandiflora* trees that are from Eberhart building in North Carolina and Sikes hall in South Carolina botanic garden pollen were observed to be exclusively bicellular. This observation shows that polymorphic pollen production by *M. grandiflora* is not a local phenomenon but is widespread across locations in the United States and Porto (Table 3. 2; Figure 3.1).

Table 3.2: Pollen germ cell number in *Magnolia grandiflora* from different sources. *Magnolia grandiflora* pollen was provided from different trees at different locations in the USA and Porto. Out of the twelve different individuals sampled ten showed polymorphic pollen, while two samples showed exclusively bicellular pollen. The number of pollen grains scored per sample was ≥ 100. Abbreviations: PM, polymorphic, II, bicellular pollen; UNCG, University of North Carolina Greensboro; SCBG, South Carolina Botanic Garden, MSU; Mississippi State University; ABG, Atlanta Botanical Garden.

Species Name	Tree	%TCP(n)	Pollen Type	Source
M. grandiflora	A	21	PM	Porto
M. grandiflora	A	17.2	PM	Petty Building,UNCG
M. grandiflora	B	3.7	PM	Petty Building,UNCG
M. grandiflora	A	2.91	PM	Eberhart Building,UNCG
M. grandiflora	B	1.45	PM	Eberhart Building,UNCG
M. grandiflora	C	0	II	Eberhart Building,UNCG
M. grandiflora	A	11.2	PM	SCBG,Clemson
M. grandiflora	B	2.7	PM	SCBG,Clemson
M. grandiflora	C	0	II	SCBG,Clemson
M. grandiflora	A	2.6	PM	Sikes Hall,Clemson
M. grandiflora	A	1.6	PM	MSU,Starkville
M. grandiflora	A	2.3	PM	ABG,Georgia

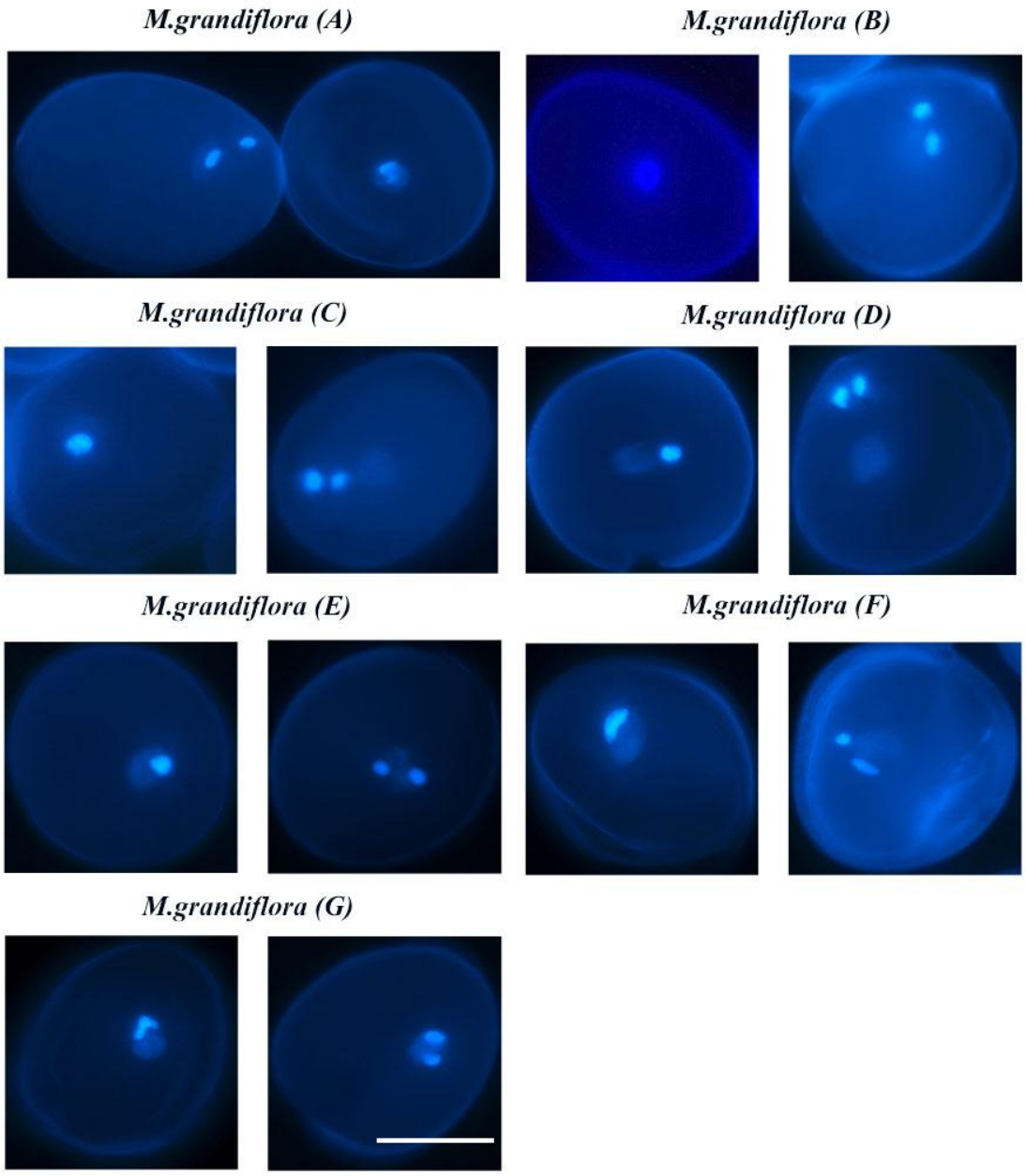


Figure 3.1: Images of *Magnolia grandiflora* bicellular and tricellular pollen types. Mature *M. grandiflora* samples pollen which are analyzed from different countries was stained with DAPI and their pollen types were observed with epifluorescent microscopy; (A) Atlanta botanical garden, (B) Belgrave Hall and Gardens, (C) University of Leicester Botanic Garden, (D) Mississippi State University, (E) University of North Carolina Greensboro (F) Porto, and (G) South Carolina Botanic garden. Scale bar =10 μm

3.3.2. Investigation of tricellular pollen type in Magnoliaceae species

After investigation of the polymorphic pollen populations, which showed the presence of some tricellular grains among a majority of bicellular pollen grains in *M.grandiflora*, the presence of both bi- and tricellular pollen nuclei in other species in the Magnoliaceae were further investigated, including *Liriodendron* and *Magnolia* species. The University of Leicester Botanic Gardens collection contained six magnolia species; *Magnolia salicifolia, Magnolia stellata, Magnolia seiboldii, Magnolia wilsonii, Magnolia campbellii, and Magnolia cylindrica*. Fully opened magnolia flowers were used to collect pollen from March to end of the April. Pollen from dehisced anthers was collected and stained with DAPI. The pollen nuclei were observed with epifluorescent microscopy and counts were made for each species. Low percentages of tricellular pollen were observed from pollen samples of *M. salicifolia, M. seiboldii* and *M. wilsonii* 2 %, 1.1 %, and 2 % respectively. *M. cylindrica* showed the highest percentage (8.9% tricellular) from the Leicester botanic garden magnolia species. In contrast, *M. campbellii* showed exclusively bicellular pollen (Table 3). During that time, Dr. Richard Gornall, Senior Lecturer and Director of the University of Leicester, Botanic Garden, , provided six herbarium specimens from the Natural History Museum in London. Herbarium samples were fixed for 24 hours in 3:1 ethanol-acetic acid. Material was transferred to DAPI solution and pollen nuclei were visualized on a Nikon eclipse 80i microscope. All herbarium samples were analysed for their pollen type but only five samples were scorable. The remaining sample, *M.macrophylla*, could not be observed because pollen was collapsed and did not stain clearly with DAPI. The analysed species, *M. virginiana, M. tamaulipana, M. macrophylla ashei, M. tripetala, and M. fraseri*. *M. macrophylla ashei* was found to possess polymorphic pollen with about 10.6 % tricellular pollen, but total pollen counts were lower than one hundred. The rest of the samples pollen were found exclusively bicellular. The investigation of various magnolia species has shown that their pollen type is not exclusively bicellular. Especially, UoL botanic garden samples, of which five out of six samples were shown to produce pollen polymorphic for germ cell number. Moreover, these findings suggested that it would be of interest to source pollen from fresh Magnolia species from China and the United States because these two countries host majority of the native Magnoliaceae family

species.

Dr. Xu Fengxia who is a Professor and Dr. Yan-Feng Kuang who is a post-doc in the South China Botanic Garden at the Chinese Academy of Science provided twenty different species in five genera: *Liriodendron, Magnolia, Michelia, Talauma and Manglietia*. *Michelia foveolata* and Talauma *hodgsonii* stamens were affected by fungus and also indehiscent, so that they were not scorable. Six species, *Liriodendron chinensis*, *Liriodendron tulipifera*, *Magnolia baillonii*, *Magnolia paeneatalauma*, *Michelia crassipes* and *Michelia yunnansis* out of eighteen scored had exclusively bicellular pollen. However, twelve species were observed to show polymorphic pollen. Overall, 1% (*Michelia figo*) to 11% (*Magnolia tripetala*) of tricellular pollen was scored in different individual stamens from same flower.

This research has shown that more than half of the analysed Magnoliaceae genera produce polymorphic pollen (Table 3.3). Further exploration of botanic gardens' websites identified more Magnoliaceae family species. Approximately ten botanic gardens director or curator's were contacted to obtain fresh magnolia species stamens and three of these , Brooklyn, San Francisco and South Carolina botanic garden, responded and were able to provide samples. These are listed below which resulted in the receipt of fifteen magnolia species stamens.

Dixie Z. Damre who is a curator in the Clemson University herbarium provided four different magnolia species, *Magnolia virginiana L.var.australis Sarg, Magnolia tripetala L., Magnolia macrophylla Michx and Magnolia macrophylla Michx.var ashei Weath* from Schoenike Arboretum and Hayden conference centre in South Carolina botanic garden, Clemson (SCBGC). David Kruse-Pickler who is an Associate Curator in San Francisco Botanical Garden supplied six Magnolia species, *Magnolia cavaleriei, Magnolia doltsopa, Magnolia floribunda, Magnolia laevifolia, Magnolia liliiflora and Magnolia maudiae* from San Francisco botanical garden, California. Tony Morosco who is a director of living collections management in Brooklyn Botanic Garden, supplied different four Magnolia species, *Magnolia denudata, Magnolia kobus, Magnolia virginiana and Magnolia virginiana var.australis* from Brooklyn Botanic Garden, New York. Richard Figlar who is a former Magnolia Society president and a magnolia expert provided *Magnolia delavayi* stamen from his garden, South Carolina.

The results of this investigation show that seven Magnolia species, *Magnolia cavaleriei*, *Magnolia denudata*, *Magnolia floribunda*, *Magnolia maudiae*, *Magnolia virginiana*, *Magnolia virgiana var australis* and *Magnolia virginiana L.var.australis Sarg* had totally bicellular pollen type. The remainder of the Magnolia species examined had polymorphic pollen. Another important genus is *Liriodendron*, which has two species *Liriodendron tulipifera* and *Liriodendron chinense* in the family Magnoliaceae. North America is host to *Liriodendron tulipifera* and China and Vietnam are host to *Liriodendron chinense*. Prof. David Twell provided *Liriodendron tulipifera* (Tulip tree) flowers from Cossington, Leicestershire to observe its pollen type. Interestingly, the total pollen counts showed 20% of tricellular pollen, which is one of the highest proportion of tricellular pollen observed in Magnoliaceae species examined. However, *Liriodendron tulipifera*, and *Liriodendron chinense* pollen, received from South China Botanic Garden, showed exclusively bicellular pollen in one flower sample. Another conflicted result involved observation from *Magnolia denudata* pollen type analysis. The first observation of *Magnolia denudata* pollen, which was collected from South China Botanic Garden showed 3.8% tricellular pollen. In contrast, another *Magnolia denudata* stamen sample was provided from Brooklyn botanic garden which was exclusively bicellular in two individual counts. The last conflicted observation was in *Magnolia mulunica* that was collected South China Botanic Garden twice in three years. In the first count that was more than six hundred pollen grains showed 0.3% tricellular pollen. Conversely, counts of the most recent independent collection (2014) from *Magnolia mulunica* were found show exclusively bicellular pollen. However, the second count was one third (n=200) of the first count so limiting the sensitivity of detection. Taken together, these results suggest that some Magnolia species show different degrees of polymorphism or pollen type in different time and locations. (Table 3.3;Figures 3.2- 3.5).

Table 3.3: Investigation of pollen germ cell number in Magnoliaceae species. Forty Magnoliaceae species of the genera *Liriodendron* and Magnolia were observed. Twenty-seven species were observed to produce polymorphic pollen and thirteen species bicellular pollen ($n \geq 100$) TCP; Tricellular pollen, PM; Polymorphic, UOLBG; University of Leicester Botanic Garden, SCBG; South China Botanic Garden, SCBGC; South Carolina Botanic Garden Clemson, KBG; Kunming Botanical Garden, SFBG; San Francisco Botanical Garden, NYBG; New York Brooklyn Botanic Garden, NHM; Natural History Museum. Species are listed in alphabetical order.

Species Name	%TCP(n)	Pollen Type	Source
Liriodendron chinensis	0	II	SCBG
Liriodendron tulipifera	0.0/20.0	II / PM	SCBG/UOL
Magnolia albosericea	4.2	PM	SCBG
Magnolia baillonii	0.0	II	SCBG
Magnolia campbellii	0.0	II	UOLBG
Magnolia cavaleriei var.platypetala	0.0	II	SFBG
Magnolia championii	5.4	PM	SCBG
Magnolia coco	4	PM	SCBG
Magnolia cylindrica	8.9	PM	UOLBG
Magnolia delavayi	2.0	PM	SCBGC
Magnolia denudata	0.0/3.8	II/PM	NYBG/SCBG
Magnolia doltsopa	2.9	PM	SFBG
Magnolia floribunda	0.0	II	SFBG
Magnolia fraseri	0	II	NHM
Magnolia henryi	4.2	PM	SCBG
Magnolia kobus	5.5	PM	NYBG
Magnolia laevifolia	4.6	PM	SFBG
Magnolia liliiflora	7.4	PM	SFBG
Magnolia macrophylla Ashei	10.63	PM	NHM
Magnolia macrophylla Michx	2	PM	SCBGC
Magnolia macrophylla Michx.var ashei Weath	0.4	PM	SCBGC
Magnolia maudiae	0	II	SFBG
Magnolia mulunica	0.0 / 0.3	II / PM	SCBG
Magnolia paeneatalauma	0.0	II	SCBG
Magnolia salicifolia	2.0	PM	UOLBG
Magnolia seiboldii	1.1	PM	UOLBG
Magnolia shangsiensis	1.1	PM	SCBG
Magnolia stellata	5.5	PM	UOLBG
Magnolia tamaulipana	0.0	II	NHM
Magnolia tripetala	1.1/11.0	PM/PM	SCBGC,KMBG
Magnolia virginiana	0.0/0.0	II/II	NHM/NYBG
Magnolia virgiana var australis	0.0	II	NYBG
Magnolia virginiana L.var.australis Sarg	0	II	SCBGC
Magnolia wilsonii	2.0	PM	UOLBG
Manglietia kwangtungensis	2.9	PM	SCBG
Michelia alba	6.5	PM	SCBG
Michelia crassipes	0.0	II	SCBG
Michelia figo	1.0	PM	SCBG
Michelia sirindhoniae	1.6	PM	SCBG
Michelia yunnansis	0.0	II	SCBG
Magnolia wilsonii	2	PM	UOLBG
Manglietia kwangtungensis	2.9	PM	SCBG
Michelia alba	6.5	PM	SCBG
Michelia crassipes	0	II	SCBG
Michelia figo	1	PM	SCBG
Michelia sirindhoniae	1.6	PM	SCBG
Michelia yunnansis	0	II	SCBG

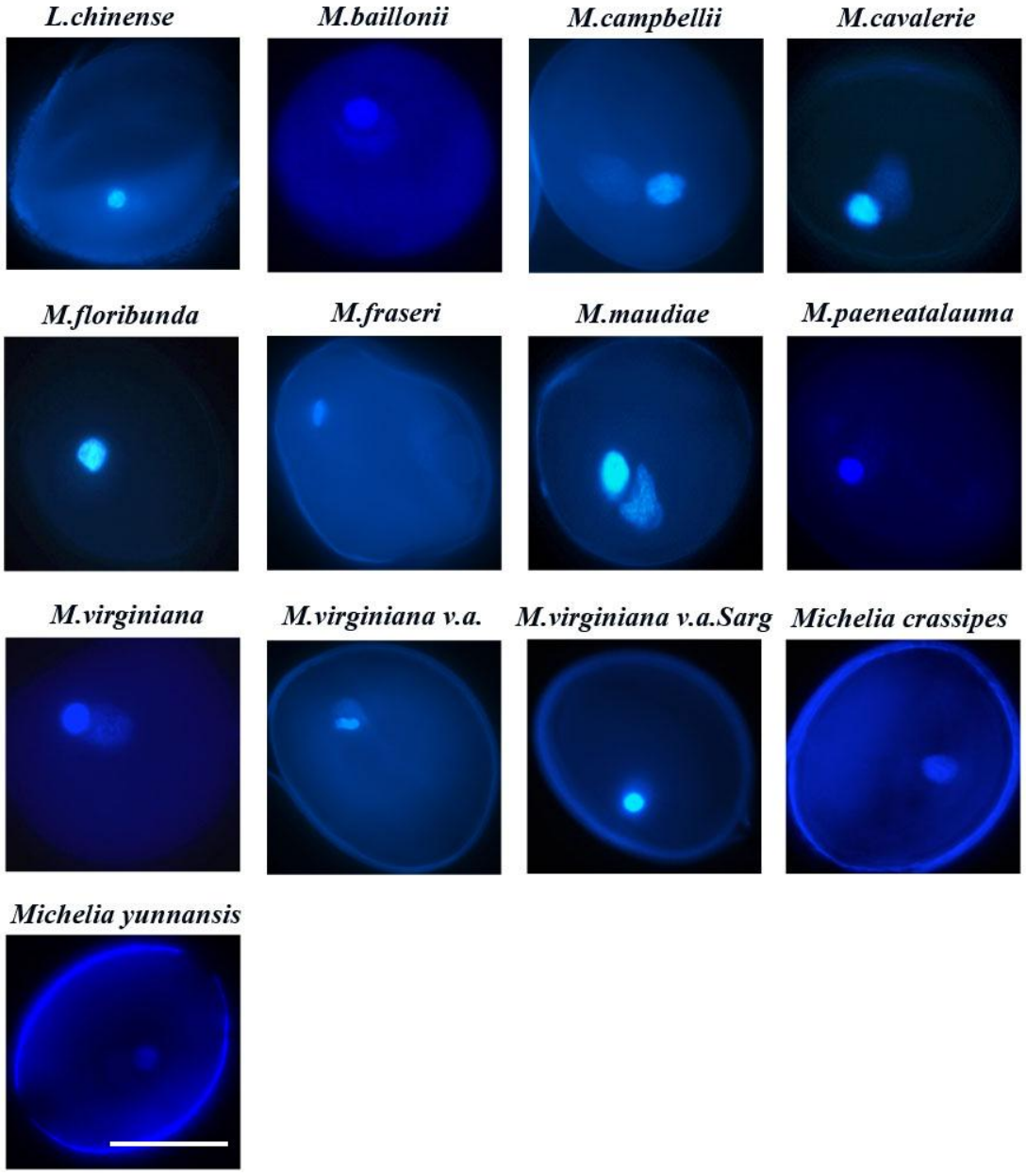


Figure 3.2: Images of bicellular Magnolia species pollen. Thirteen species were observed with exclusively bicellular pollen. Scale bar =10 μm

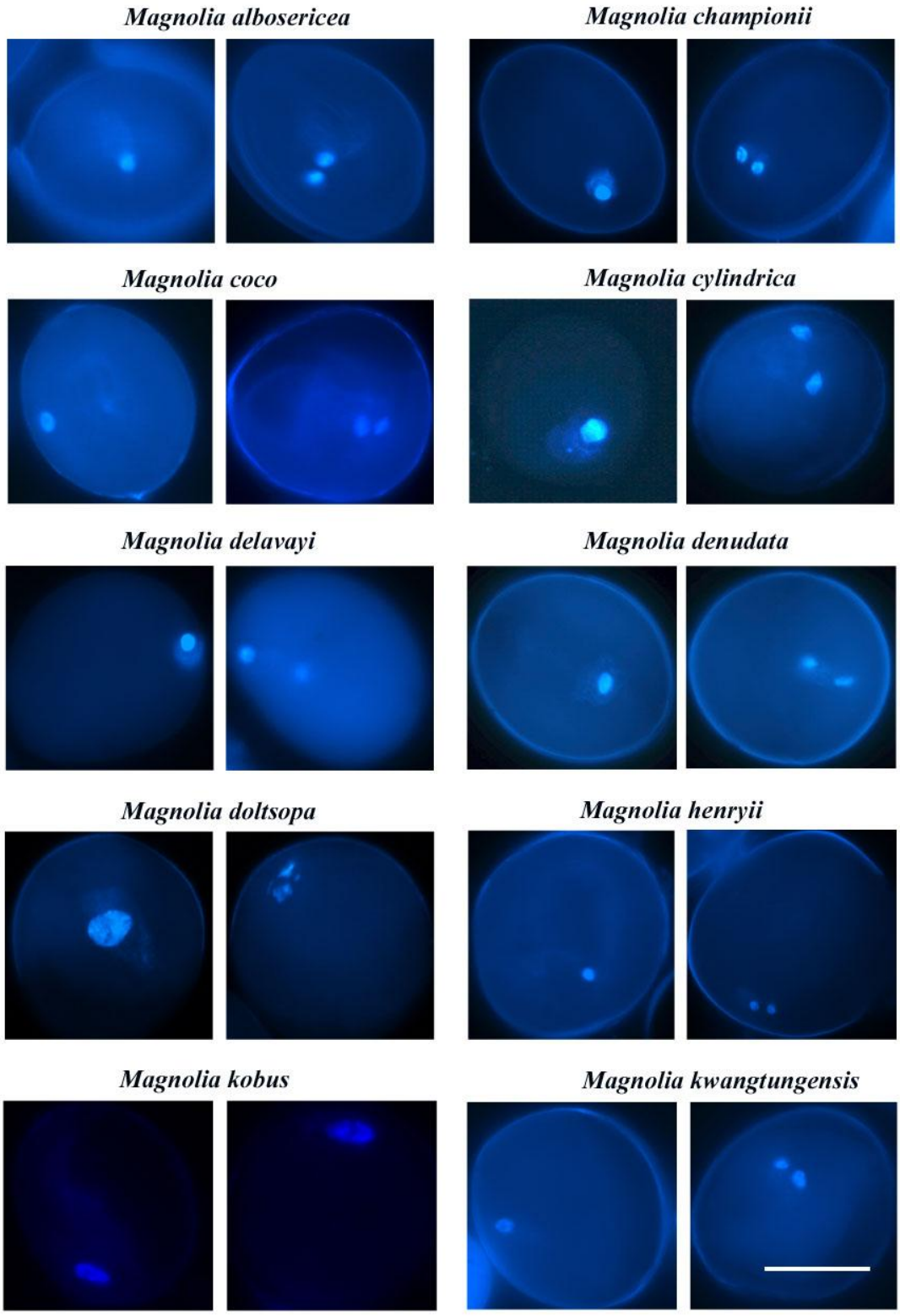


Figure 3.3: Images of bicellular and tricellular pollen of Magnolia species. Pollen was stained with DAPI. Bicellular and tricellular pollen images were captured at 60X magnification for each species. Scale bar =10 μm

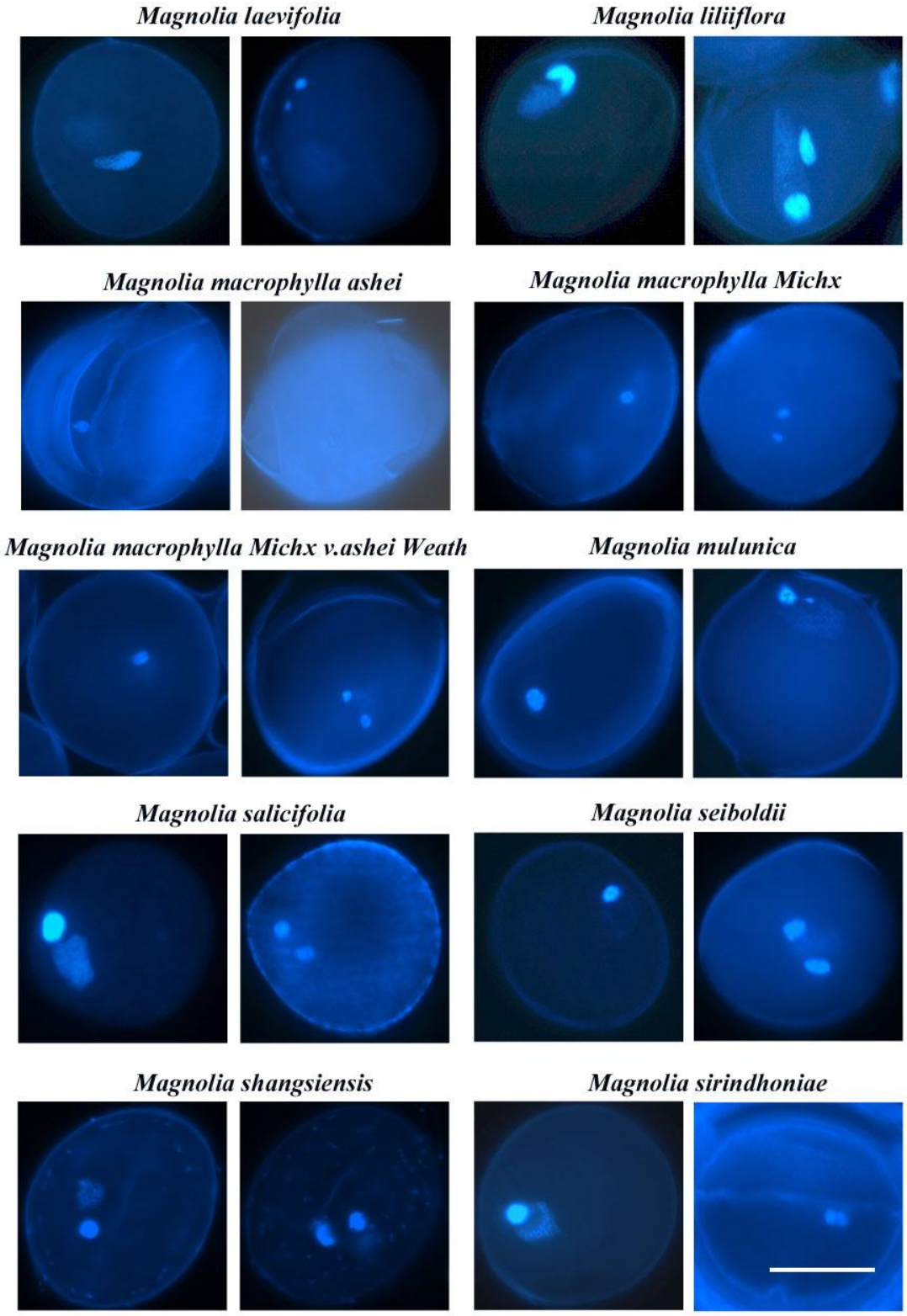


Figure 3.4: Images of bicellular and tricellular pollen of Magnolia species. Pollen was stained with DAPI. Bicellular and tricellular pollen images were captured at 60X magnification for each species. Scale bar =10 μm

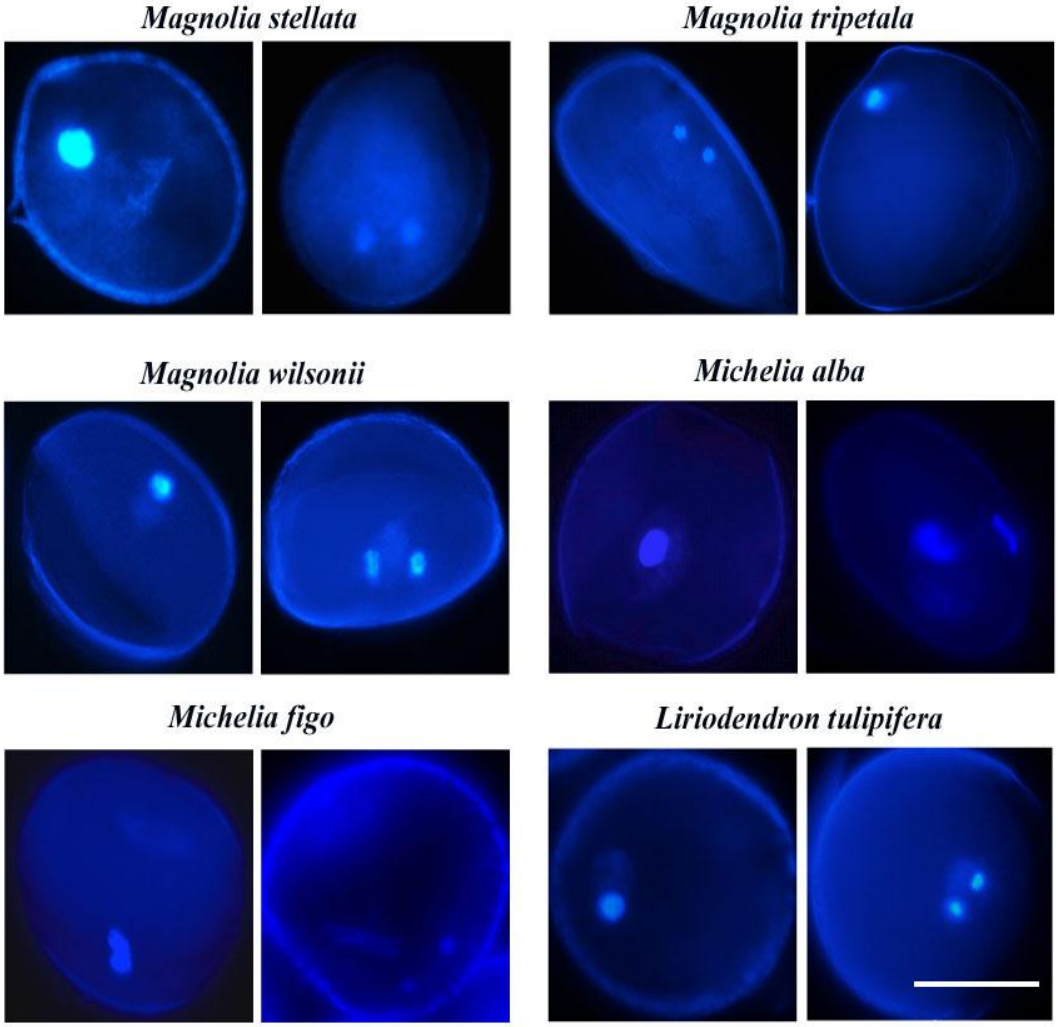


Figure 3.5: Images of bicellular and tricellular pollen of Magnolia species. Pollen was stained with DAPI. Bicellular and tricellular pollen images were captured at 60X magnification for each species. Scale bar =10 μm

3.3.3. Temperature treatment of *Magnolia grandiflora*

Several factors, both internal and external, determine the number of nuclei present in pollen. In particular, temperature is known to impact upon reproductive activities (Vara Prasad *et al.*, 2000; Sato *et al.*, 2002) and the duration of meiosis (Bennett, 1977). In addition, temperature affects the lifespan and performance of pollen (Delph *et al.*, 1997; Hedhly *et al.*, 2005) as well as the duration of mitosis (Klindworth & Williams, 2001). Recent studies have shown that environmental conditions favouring high humidity and aquatic temperature for generative cell division favours the production of tricellular pollen at anthesis in *Annona cherimola* (Lora *et al.*, 2009). In an attempt to determine whether or not the temperature to which pollen is exposed during development has any impact on the number of nuclei present, Lora *et al*. (2009) examined the nuclei of *A. cherimola* pollen grains during different developmental stages at varying temperature levels. This revealed that both tricellular and bicellular pollen was released. In order to examine whether temperature could affect the number of nuclei in pollen grain, we determined the number of nuclei of *M. grandiflora* pollen grains during progressive stages of pollen development at different temperatures in controlled growth chambers. This study investigates the coexistence of bicellular and tricellular pollen in *Magnolia grandiflora* at anther dehiscence in material collected Beaumont Hall in the University of Leicester Harold Martin Botanic Garden, Leicester, United Kingdom.

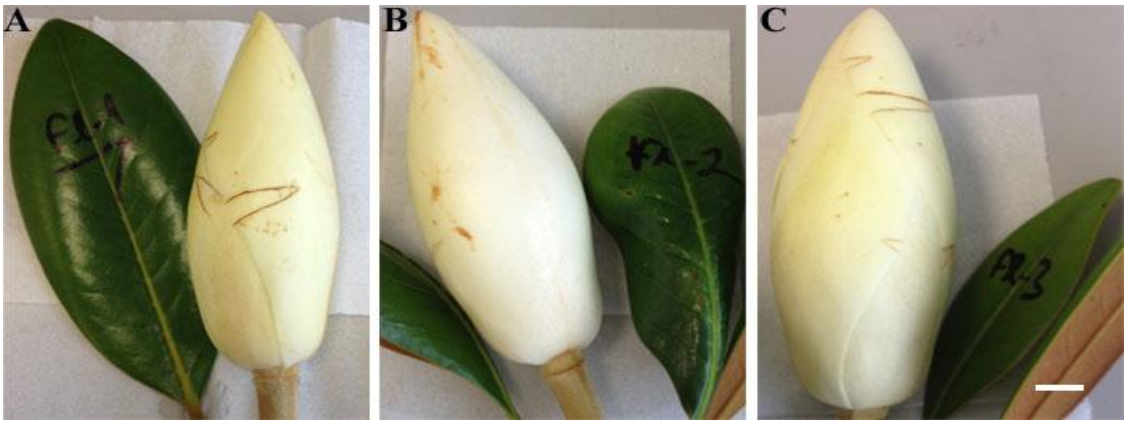


Figure 3.6: Three unopened *Magnolia grandiflora* flower buds with pedicels. These flowers were labelled A; Flower-1, B; Flower-2 and C; Flower-3 and their lengths were measured 11 mm, 11.5 mm and 13.5 mm respectively. Scale bar =10 μm

The experiment was performed during the flowering period in September. To investigate the effect of temperature on the percentage of tricellular pollen and rate of pollen development in *Magnolia grandiflora*, three unopened flower buds with pedicels collected from same *M.grandiflora* tree in September.

Figure 3.7: Design of the temperature treatment for *Magnolia grandiflora* pollen experiment. Unopened bud petals were cut to take stamens using a razor blade (A). Stamens were removed and put in plastic petri dishes, which were each labelled with the temperature regime and flower number (B). Petri dishes were sealed with filter tape and put in the selected growth chambers with min/max thermometer and humidity meter (C). Scale bar =10 μm

Pollen from three flowers, labelled flower-1, flower-2, and flower-3, were counted in order to determine their proportion of tricellular pollen during the experiment. The research question proposed was, does temperature play a role in the rate of pollen development in *Magnolia grandiflora*? Four different petri dishes labelled 15 ^{o}C, 20 ^{o}C, 25 ^{o}C, and 30 ^{o}C were prepared with wet tissues and sealed filter tape for each of flowers. Ten stamens were put in petri dishes from each flower and temperature treatment. Overall 120 stamens were put into a total of 16 plastic petri dishes in four temperature regimes. Every 24 hours one stamen was taken from each labelled petri dishes. Then, collected stamens were treated with fixative immediately. This experiment was performed until stamen dehiscence occurred under all temperature treatments. The experiments were carried out in four identical growth chambers, set at 15 ^{o}C, 20 ^{o}C, 25 ^{o}C, and 30 ^{o}C with continuous light.

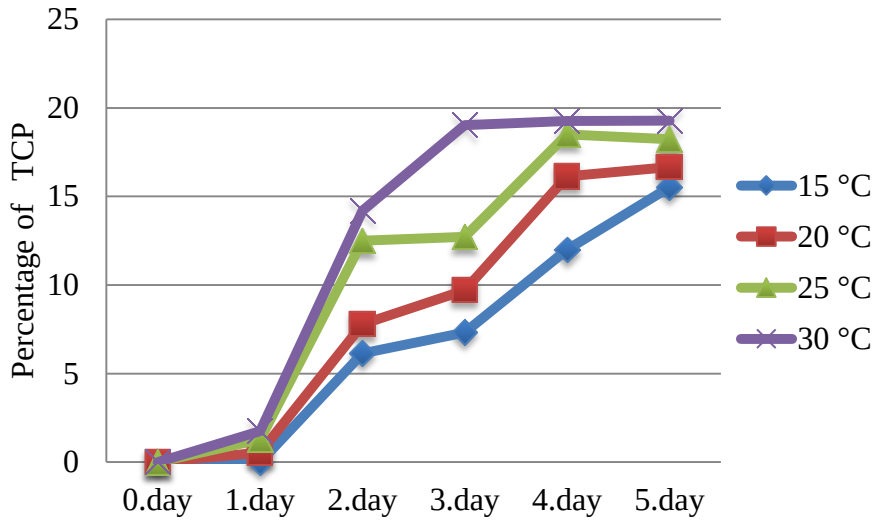


Figure 3.8: Percentage of tricellular pollen in stamens from *M. grandiflora* flower-1, cultured at different temperatures. During a period of 5 days, stamens were taken and fixed, then pollen phenotype was scored. After 3 days, stamens, which were in the 30^{o}C growth chamber were dehiscent and showed the highest percentage of tricellular pollen (19.0%-19.3%). After 4 days, stamens were dehiscent in the 20 ^{o}C and 25 ^{o}C conditions. Lastly, 15^{o}C cabinet stamens were dehiscent. Besides, the lowest percentage of tricellular which is 15.5% was observed after fives days. Once pollen was shed, the percentage of tricellular pollen showed the same trend.

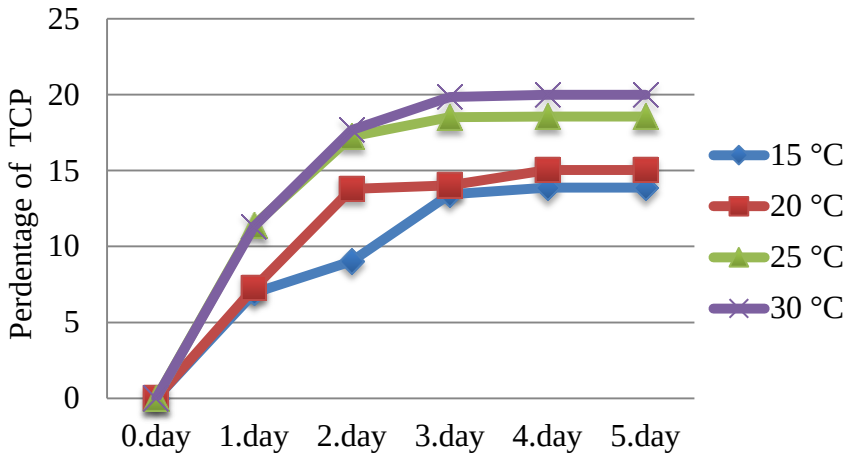


Figure 3.9: Percentage of tricellular pollen in stamens from *M. grandiflora* flower-2, cultured at different temperatures. During a period of 5 days, stamens were taken and fixed, then pollen phenotype was scored. . Three days later, stamens, which were in the 25 ^{o}C and 30 ^{o}C conditions were dehiscent and showed the highest percentage of tricellular pollen (19.8-20%). After 4 days, stamens were dehiscent in the 15^{o}C and 20 ^{o}C conditions. Likewise, the lowest percentage of tricellular pollen, which is 13.9%, was observed after 5 days. Once pollen was shed, the percentage of tricellular pollen showed the same trend.

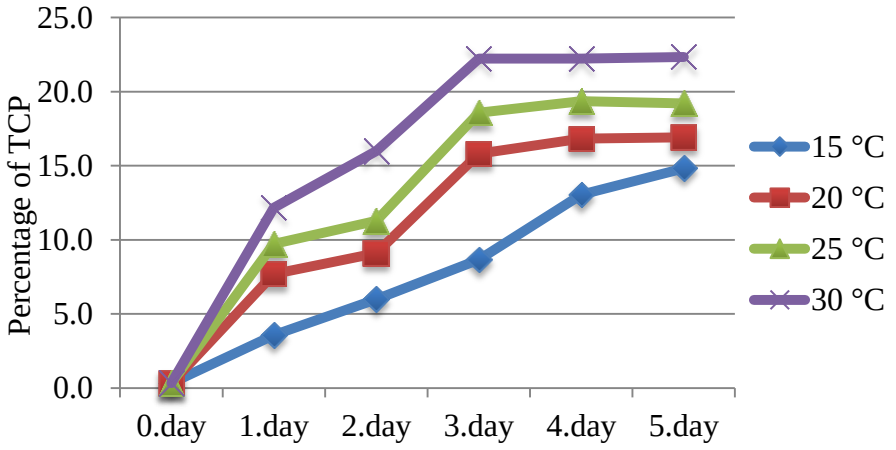


Figure 3.10: Percentage of tricellular pollen in stamens from *M. grandiflora* flower-3, cultured at different temperatures. At the beginning of the experiment flower-3 stamens showed some (0.3 %) tricellular pollen. After 3 days stamens, under 20^{o}C, 25^{o}C and 30^{o}Cconditions, were dehiscent the highest percentage of tricellular pollen (22%) was observed in 30^{o}C conditions. After 5 days, stamens were dehiscent in the 15^{o}C growth chamber. Also, the lowest percentage of tricellular which is 14.8%, was observed after 5 days. When pollen was shed, the percentage of tricellular pollen showed the same trend.

In summary, the observation of pollen from stamens of three different flowers in same *M. grandiflora* was designed and analysed as follows; at the beginning of the experiment three unopened flowers stamens were taken off separately. Flower-3 unopened bud was longer than Flower-1 and 2. Moreover, 2-3 stamens were taken from each flower. The numbers of pollen were counted from each of day collection. To fix anthers from *Magnolia grandiflora*, freshly prepared fixative was used. The fixative made by adding 1 volume of glacial acetic acid and 3 volumes of % 100 ethanol. Collected anthers were fixed every 24 hours. Stamens were put on the microscope slide with 5 µl of DAPI solution (4', 6-diamidino-2 phenylindole). Pollen grains were released from each of stamen on the microscope slide using by hypodermic syringe and dissecting needle under the dissecting microscope. Then, pollen grains were gently pressed down with a cover slip to flatten the sample, sealed with nail varnish to observe the number of pollen nuclei and counts were scored as a zero day (0.day) result. The 0.day result showed that flower-1 and flower-2 have exclusively bicellular pollen type; flower-3 has very rare tricellular pollen type, which is 0.3%. After one day, flower-1 stamen pollen showed very low percentage of tricellular pollen that is from 0% to 1.8%, in four different temperature regimes.

However flower-2 and 3 were showed increased tricellular pollen type, which is 11.3% and 12.2% respectively in the 30^{o}C chamber. Moreover, three days later all flower stamens anther dehiscence in the 30^{o}C growth chambers, also they showed the highest percentage of tricellular pollen, about 19 %, 20% and 22.3%, in flower-1, 2 and 3 correspondingly. However, at 15^{o}C flowers stamens dehisce five days later. In addition, the lowest percentages of tricellular pollen were observed (Figure 3.8-3.10). These findings suggest that in general, high temperature regimes increase the rate of pollen development in *M. grandiflora*. Furthermore, the percentage of tricellular pollen was increased until anther dehiscence occurs and the highest proportion of tricellular pollen was observed when pollen was shed. After anther dehiscence there was no further increase in the effect of temperature on the percentage tricellular pollen. Taken together, these results suggest that counting twice further from flower-1 and flower-2, was required to test the results statistically, because, they have approximately the same bud lengths. Flower-3 was eliminated because it was longer than flower-1 and 2. The experiment was performed using the same approach. However, 2 more stamens were

taken for each day from flower-1 and 2 to obtain three counts in total.

Statistical analysis was performed using statistical software GraphPad Prism 6.0. Statistical analysis of the percentage of tricellular pollen for each flower was undertaken using a two-way ANOVA (analysis of variance) test, which evaluated differences in the examined various temperature conditions and time (days). Two-way ANOVA shows a significant association between the number of tricellular pollen grains and the temperature if we adjust for time (day) and flower. In order to determine difference data sets in multiple comparisons Tukey's multiple comparison test used for each flower temperature treatment.

Table 3.4: Tukey's multiple comparison test result for flower-1. The percentage of tricellular pollen at each temperature was tested and compared pairwise to determine significant differences. According to p-value calculation, 15 ^{o}C vs. 20 ^{o}C, 20 ^{o}C vs. 25 ^{o}C, and 25 ^{o}C vs. 30 ^{o}C showed no significantly difference from each other. Nevertheless, 15 ^{o}C vs. 25 ^{o}C, 15 ^{o}C vs.30 ^{o}C, and 20 ^{o}C vs. 30 ^{o}C showed various levels of significant differences. P-value<0.05, ns: not significant

Temperature	Significant?	Summary	Adjusted P- Value
15^{o}C vs. 20^{o}C	No	ns	0.3165
15^{o}C vs. 25^{o}C	Yes	*	0.0195
15^{o}C vs. 30^{o}C	Yes	***	0.0007
20^{o}C vs. 25^{o}C	No	ns	0.4166
20^{o}C vs. 30^{o}C	Yes	*	0.0226
25^{o}C vs. 30^{o}C	No	ns	0.3511

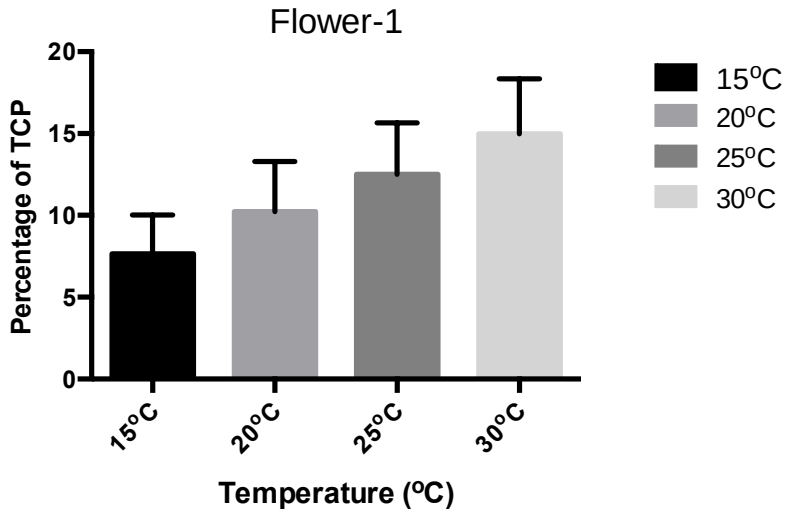


Figure 3.11: Effect of the temperature on *M. grandiflora* flower-1 pollen development. After 5 days, three stamens were fixed for each temperature treatment. Pollen was stained with DAPI and phenotype was scored under the microscope. Mean of the tricellular pollen for 15 ^{o}C, 20 ^{o}C, 25 ^{o}C, 30 ^{o}C temperature in 5 days were: 7.6%, 10.2%, 12.5%, and 15.0% respectively. Error bars indicate standard error of the mean for each individual. N=3.

Table 3.5: Tukey's multiple comparison test result for flower-2. Each temperature was tested pairwise against each other to determine significant differences. According to p-value calculation, 15 ^{o}C vs. 20 ^{o}C, and 25 ^{o}C vs. 30 ^{o}C showed no significant differences from eachother. However, 15^{o}C vs. 25^{o}C, 15 ^{o}C vs. 30^{o}C, and 20 ^{o}C vs. 30 ^{o}C showed various level of significant differences. P-value<0.05, ns: not significant

Temperature	Significant?	Summary	Adjusted P- Value
15^{o}C vs. 20^{o}C	No	ns	0.1824
15^{o}C vs. 25^{o}C	Yes	***	0.0005
15^{o}C vs. 30^{o}C	Yes	****	< 0.0001
20^{o}C vs. 25^{o}C	Yes	*	0.0337
20^{o}C vs. 30^{o}C	Yes	**	0.0026
25^{o}C vs. 30^{o}C	No	ns	0.5684

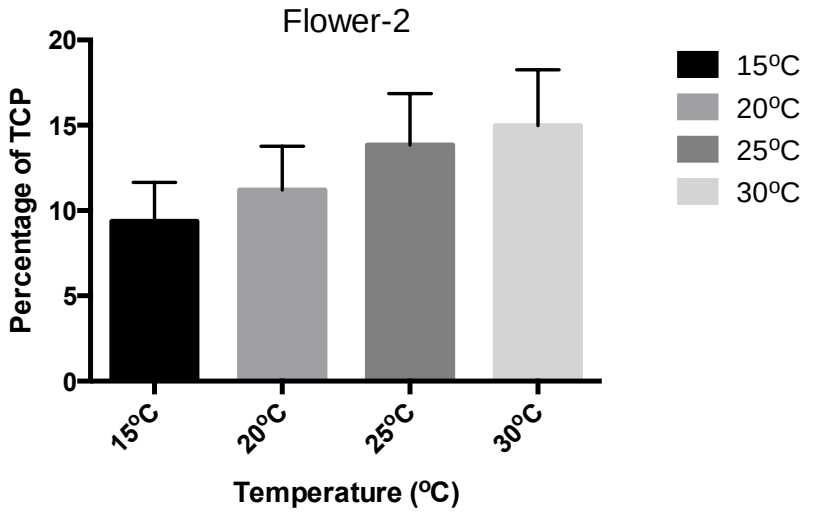


Figure 3.12: Effect of the temperature on *M. grandiflora* flower-2 pollen development. After 5 days three stamens were taken and fixed for each temperature treatment. Pollen was stained with DAPI and phenotype was counted. The means of the percentage tricellular pollen for 15 °C, 20 °C, 25 °C, 30 °C temperature at five days was 9.3%, 11.2%, 13.8% and 14.9% respectively. Error bars indicate standard error of the mean for each individual. N=3

3.4. Discussion

The main goal of the research described in this chapter was to document and investigate the coexistence of tricellular pollen in Magnoliaceae. In addition, a secondary aim was to investigate the effect of temperature on pollen development rate and tricellularity in *Magnolia grandiflora*. The investigation of pollen types in Magnoliaceae species has shown that more than sixty percent of the species analysed showed polymorphic pollen types. Moreover, observation of the polymorphic pollen phenotype is widespread and confirmed in more than forty Magnolia species sourced from America, Asia and Europe. For instance, the highest percentage of tricellular, pollen of approximately 20%, was observed in *Magnolia grandiflora*, and *Liriodendron tulipifera*. This study is the largest so far documenting and analysing Magnoliaceae family pollen type with respect to germ cell number. The most obvious finding to emerge from this research is that 68% of species analysed in the Magnoliaceae showed polymorphic pollen in contrast to the report of Brewbaker (1967), who reported Magnoliaceae species are exclusively of the bicellular pollen type. Furthermore, the highest temperature regimes gave rise to a significantly earlier rate of pollen development and percentage of

tricellular pollen in *M. grandiflora* demonstrating that temperature affects the rate of pollen development including generative cell division to form the sperm cells.

The dehiscent anthers of approximately 70% of angiosperm species contain bicellular pollen. An evolutionary derivative of bicellular pollen is tricellular pollen, which has been identified in a number of plant families (Brewbaker, 1967). By comparison to the observations made by Brewbaker during anther dehiscence, this study has found that in 27 out of 41 Magnoliaceae species examined contained polymorphic pollen. It could thus be surmised that the bicellular pollen type is dominant in species belonging to the Magnoliaceae family and in every basal angiosperm (Brewbaker, 1967; Williams et al., 2014). By contrast, it is only rarely that bicellular and tri-cellular pollen grains appear in the same genus (Brewbaker, 1967). Apart from Brewbaker, only a handful of other studies have reported the co-existence of the two pollen types in the same plant during anther dehiscence. Generally, such co-occurrence of pollen types has been observed in relatively old order species, including Laurales (Sampson, 1969; Gardner, 1974) and Alismatales (Grayum, 1985), in species of the families Olacaceae (Santalales) (Johri et al., 1992) and Euphorbiaceae (Malpighiales) (Webster and Rupert, 1973), as well as in contradictory studies of species of different genera like *Capsicum* (Lengel, 1960), *Populus* (Hamilton and Langridge, 1976), Diospyros (Sugiura et al., 1998), Annoneceae (Lora et al., 2009), and *Saxifraga* (Zhang and Gornall, 2011). The present study detected polymorphic and bicellular pollen in *M. grandiflora* from different trees located in the same area. This suggests that, in addition to climate and location, pollen types in *M. grandiflora* may also be affected by genetics. Furthermore, pollen types also differ among same species native to different countries, for example, compared to the *Liriodendron tulipifera* collected from Leicester,and that collected from South China Botanic Garden had solely bicellular pollen. Disparate results can also be obtained with different collections from the same place. For instance, over a period of three years, *Magnolia mulunica* was collected twice from the South China Botanic Garden. In the first collection, six hundred pollen grains contained tricellular pollen in a proportion of 0.3%. In the second collection in 2014, however, bicellular pollen was predominant in the two hundred pollen grains from *Magnolia mulunica*.. Nevertheless, these findings indicate that collection time and location significantly influence the pollen type contained in species of Magnolia.

The results of experiments involving pollen development under different temperature treatments, revealed that the amount of tricellular pollen at anthesis in *M. grandiflora* varies according to temperature. In a fluctuating environment, such temperature-regulated phenotypic plasticity in the proportion of tricellular to bicellular pollen could serve as a useful adaptation mechanism, modulating the timing of pollen access to the ovule in order to make sure that the egg cell is reached by some pollen tubes at the proper phase of development, which also lends credence to the notion that phenotypic plasticity has contributed to evolution (Pigliucci, 2005). Therefore, tricellular pollen is likely to be produced in conditions where there are high levels of humidity in the proximity of the pollen and the temperature is conducive to generative cell division. This theory still requires close assessment, but Brewbaker (1967) proposed that aquatic taxa could benefit significantly from tricellular pollen. Furthermore, with the exception of Poaceae, tricellular pollen dispersal was observed in a more hydrated mode as well (Brewbaker, 1967; Knox, 1984). These findings provide the following insights for future research. Pollen could be collected from various Magnolia species in different locations which especially China and South America (Mexico, Colombia), then analyzed for pollen germ cell number in order to increase the number of Magnoliaceae species analysed and to further confirm the reproducibility of phenomenon with environment, individuals and location. Moreover, another possible area of future research would be to map pollen germ cell number onto a cladogram of Magnoliaceae species to understand the relationship between the evolution of Magnolia species and the germ cell number character. Finally, considerably more work will need to be done to explore the details of the temperature effect on the rate of pollen development and tricellularity in *Magnolia grandiflora* especially during pollen germination e.g., sampling of pollen from different *Magnolia grandiflora* trees to be used in temperature treatments.

Chapter 4

FUNCTIONAL CONSERVATION OF DUO1 IN PLANTS

4. Functional Conservation of DUO1 in Plants

4.1. Summary

Background

The male-germline-specific R2R3 MYB transcription factor DUO1 is specifically expressed in the male germline of *Arabidopsis* and is required for germ cell division and gamete differentiation (Brownfield *et al.*, 2009a). DUO1 is present in divergent angiosperms and its significant sequence conservation and ability to complement *Arabidopsis duo1* mutants, adds weight to its essential role in angiosperm reproduction. It is of great interest to establish whether DUO1 is functionally conserved in other angiosperms (eg. rice, tomato) and to investigate the expression and function of homologs of DUO1, which are present in early land plants.

Results

The expression and function of DUO1 orthologues from *Solanum lycopersicum* (dicot), *Oryza sativa* (monocot), and *Physcomitrella patens* (moss) were investigated. Expression of SlDUO1-A and OsDUO1 and transgenes as mCherry fusion proteins was sufficient to rescue failed germ cell division in *duo1* mutants. Plants harbouring SlDUO1-B and PpDUO1-A mCherry gene fusions however did not show expression and were unable to rescue failed germ cell division in *duo1* mutants. A PpDUO1-B-mcherry fusion was expressed in some plants, but was unable able to rescue failed germ cell division in *duo1* pollen. OsDUO1, SlDUO1-A and SlDUO1-B were shown to transactivate AtDUO1 target gene promoters in transient expression assays in tobacco leaves, but PpDUO1-A and PpDUO1-B did not.. AtDUO1-, SlDUO1-A-, and OsDUO1-mCherry T2 lines also showed genetic segregation ratios consistent with complete rescue of failed transmission of *duo1* pollen.

Conclusion

DUO1 is conserved in selected species and shows preferential expression in

reproductive organs. Expression of the rice DUO1 and tomato DUO1-A orthologs are able to complement the *duo1* generative cell division and transmission defect, which demonstrates a high level of conservation of DUO1 function in *Arabidopsis*. Moss DUO1 sequences (PpDUO1-B) however do not complement *duo1* defects which may reflect their divergent functions in their native host, moss.

4.2. Introduction

DUO1 is a male germline-specific R2R3-type MYB transcription factor that is initially expressed in the newly formed generative cell or male germline following asymmetric division of the microspore (Rotman *et al.*, 2005). It is confirmed that DUO1 is a key-factor in male germline development as this transcription factor integrates male germ cell cycle progression with differentiation, resulting in the formation of a pair of sperm cells which function in double fertilization (Brownfield *et al.*, 2009a). DUO1 affects germ cell cycle progression through the control of G2/M phase-specific accumulation of *CYCB1;1* (Brownfield *et al.*, 2009a). Simultaneously, DUO1 also activates the expression of germline specific genes related to sperm cell differentiation, specifically *MGH3* (Okada *et al.*, 2005), *GEX2* (Engel *et al.*, 2005) and *GCS1/HAP2* (Mori *et al.*, 2006). Recent research has increased the number of DUO1 targets to over 15 genes (Borg *et al.*, 2011).

Regardless of the important part DUO1 plays in controlling male germline development, there is limited knowledge with regard to the other potential domains of the DUO1 protein beyond the well-known DNA-binding R2R3 MYB repeats. A characteristic that sets DUO1 apart from other R2R3-type MYBs is the existence of a supernumerary lysine residue at the start of the R3 domain (Rotman *et al.*, 2005). This characteristic is unique to DUO1 and its orthologs, as it is commonly conserved in divergent angiosperm groups from monocots to dicots. Therefore, it is logical to put forward the idea that its conservation is crucial from an evolutionary and functional perspective, but its importance has not been established empirically.

This chapter describes experiments undertaken to functionally describe the DUO1 protein by investigating the importance of the supernumerary lysine residue and

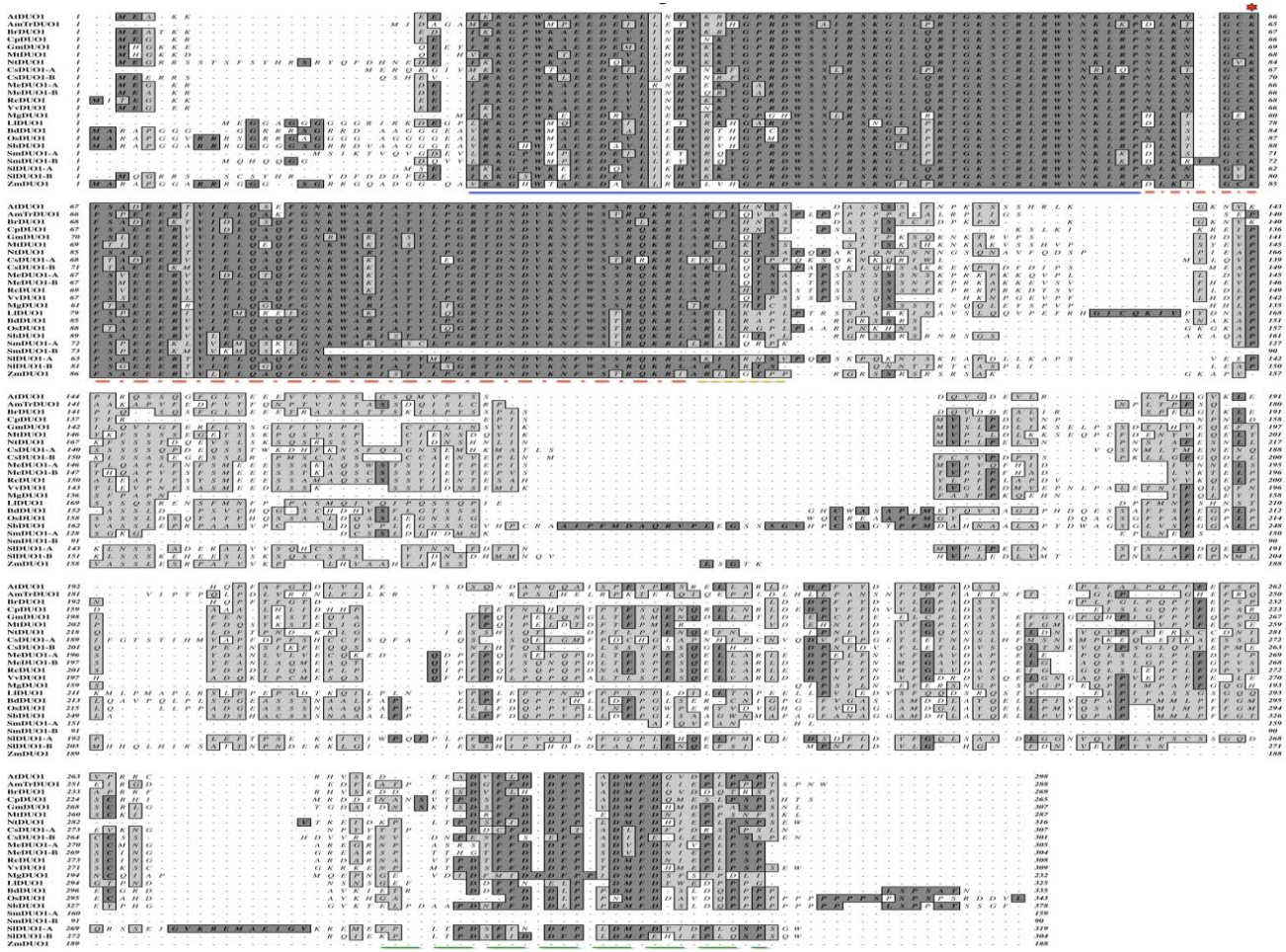
pinpoint other domains crucial to its function in plants. Initially, this chapter discusses the conservation and expression of DUO1 protein in selected plant tissues. Following this, the design of a transient luciferase assay will be employed to quantify transactivation potential within the DUO1 and be analyzed. The complementation potential of the DUO1 variants is then discussed, with the help of an in vivo pollen assay. Finally, a corroborative experiment looking into the biological function of complemented *duo1* germ cells (transmission analyses) is examined.

4.3. The *DUO1* Gene

The DUO1 protein is 297 amino acids in length and, as with other R2R3-type MYB proteins, DUO1 includes R2 and R3 domains repeated in sequence at the N-terminal portion of the protein. To try and pinpoint current characteristics and also underline other conserved regions, a ClustalW alignment was undertaken between AtDUO1 and its orthologs from a series of evolutionarily divergent species. An extensive search for DUO1 orthologs was also undertaken, with the help of data previously collated in the ensemble plant database resource, as well as conventional BLAST searches across a variety of plant databases. The orthologs series contained in the alignment were from *Arabidopsis lyrata* (lyre-leaved rock-cress), *Amborella trichipoda Brassica rapa* (turnip mustard), *Carica papaya* (papaya), *Glycine max* (soy bean), Medicago truncatula (barrel clover), *Nicotiana tabacum* (tobacco), *Cucumis sativu*s (cucumber), *Manihot esculenta* (cassava), *Ricinus communis* (castor bean), *Vitis vinifera* (common grape vine), *Mimulus guttatus* (common monkey-flower), *Lilium longiflorum* (lily), *Brachypodium distachyon* (purple false brome), *Oryza sativa* (rice), *Sorghum bicolor*, *Zea mays* (maize), *Solanum lycopersicum* (tomato) and *Selaginella moellendorffii*. *Selaginella* and tomato included two DUO1-related proteins in their genome and each protein is set apart as A and B. Even thought DUO1 orthologs in higher plants show a conservation of the spacing and identity related with the signature lysine residue, it is noteworthy that the lower land plant orthologs (moss and *Selaginella*) appear to include further additional amino acids at the location of K66 (Figure 4.1). SmDUO1-B has a further pair of amino acids present, namely tyrosine and leucine residues (Figure 4.1). All DUO1 orthologs show a stretch of highly conserved amino acids past the end of the R3 MYB repeat (Fig 4.1). In AtDUO1, the end of the R3 MYB repeat is very basic (the last

10 amino acids have >50% basic residues) and this basic nature is present throughout this conserved stretch of 9 amino acids (22% basicity). Basic regions highlight a nuclear localisation signal (NLS) (Raikhel, 1992; Lange *et al.*, 2007) and this conserved region, as a result, was thought of being worth additional research. A further highly conserved region found in all species, apart for maize, moss and *Selaginella*, is a stretch of 25 amino acids at the C-terminal tail of the proteins with 36% acidic nature and 48% hydrophobic nature (Figure 4.1).

Figure 4.1: Conservation of the DUO1 protein. The amino acid sequence of AtDUO1, and its orthologs across all plants were aligned with ClustalW alignment using MacVector. The two DNA-binding MYB domains are indicated by blue solid (R2) and red dashed line (R3) under the sequences. The supernumerary lysine (K66) residue was showed above the sequence by a red star. The basic region at the end of the R3 MYB repeat is indicated with a yellow dashed line and the acidic c-terminal domain with a green dashed line. Some species contain two DUO1-related proteins in their genome and each protein is distinguished as A and B (see next page).



4.4. Identification of DUO1 Orthologs in Plants

Solanum lycopersicum (Sl) homologs, *Oryza sativa* (Os) orthologs, and *Physcomitrella patens* (Pp) homologues to *Arabidopsis thaliana* (At) *DUO1* were identified with Blast which stands for Basic Local Alignment Search Tool (BLAST) on the Ensembl Plant, and National Center for Biotechnology Information (NCBI) database. This database list two possible orthologs of *AtDUO1* for moss, tomato and one possible orthologs of AtDUO1 in rice. Tomato DUO1 proteins, which are Solyc01g090530.1 and Solyc10g019260.1, were named as SlDUO1-A and SlDUO1-B. Rice DUO1 protein is Os04g0549500, named as OsDUO1. Moss DUO1 proteins that are Pp1S114_136V6 and Pp1S16_281V6 were named as PpDUO1-A and PpDUO1-B. Both putative tomato DUO1 orthologs have three exons and two introns like *A. thaliana* DUO1. Predicted *SlDUO1-A* is 960bp and 319aa in length, and *SlDUO1-B* 915bp and 304aa in length. Also, two tomato DUO1s are similar in sequence, having a sequence 55% idendity. Both sequences of the *Physcomitrella patens* DUO1 proteins were originally obtained from the Ensembl Plant website (http://plants.ensembl.org/). *PpDUO1*-A is 490 amino acids long that showed 36.5 % identity with AtDUO1 peptide sequence. *PpDUO1*-B has two parts. Pp1S16_281V6_1, which including exon-1, 2, and 3 is the first part of the *PpDUO1-B* gene. Pp1S16_281V6_1 is 413 amino acids long that showed 32.2 % identity with AtDUO1 peptide sequence. Pp1S16_281V6_2 (exon-4) is the second part of the *PpDUO1-B* gene and it is 208 amino acids length, which showed 26.6% similarity with *AtDUO1* amino acid sequence. However, *PpDUO1-B* designed like one gene and unknown sequence was considered as an intron. Nucleotide sequence of the *Oryza sativa DUO1* proteins were originally obtained from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). *OsDUO1* is 343 amino acids long that showed 40 % identity with *AtDUO1* peptide sequence (Figure 4.2).

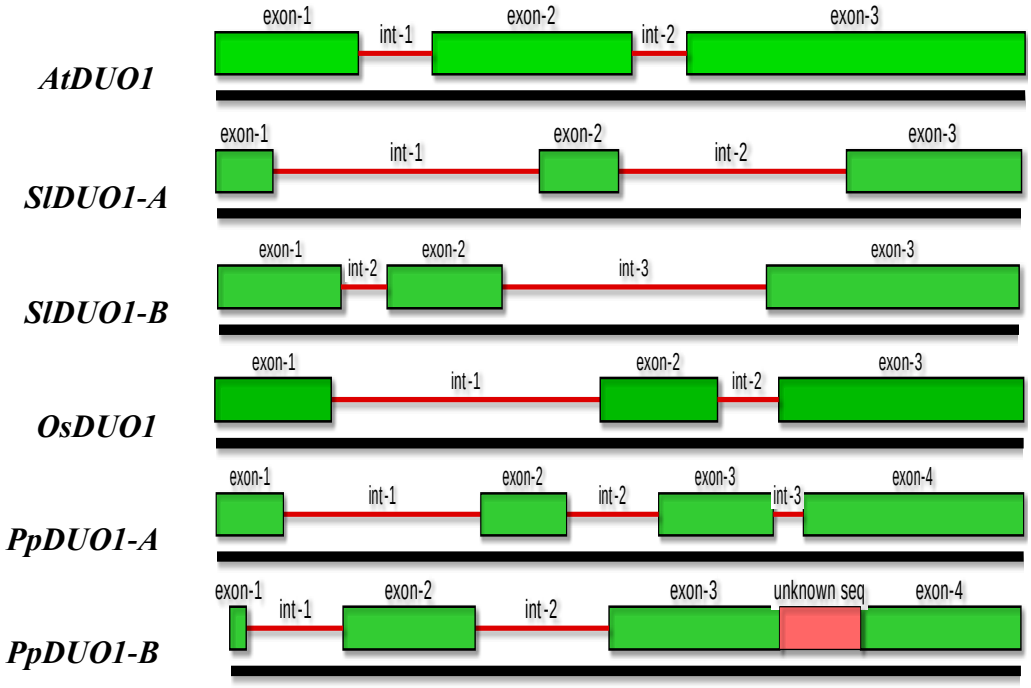


Figure 4.2: Predicted DUO1 gene structure in selected species. *DUO1* sequences 4 species which *Solanum lycopersicum* (Sl) homologs, *Oryza sativa* (Os) orthologs, and *Physcomitrella patens* (Pp) homologs to *Arabidopsis thaliana* (At), were identified using BLAST on various genome databases. The structures of the *DUO1* orthologs were mapped using MacVector software.

4.4.1. Conservation of DUO1

Through the analysis of sequence homology at the DNA level, one can identify highly conserved regions amongst orthologs genes. In the case of DUO1, the most obvious region of high homology is the R2R3 MYB DNA binding domain. Furthermore, DUO1 is unique when compared to other R2R3-type MYBs because it contains a supernumerary lysine residue highly conserved across evolutionary significant era. There is also an intron splice site within the mRNA sequence encoding the MYB domain that is also conserved. These features used to define whether sequences isolated where indeed DUO1 orthologs.

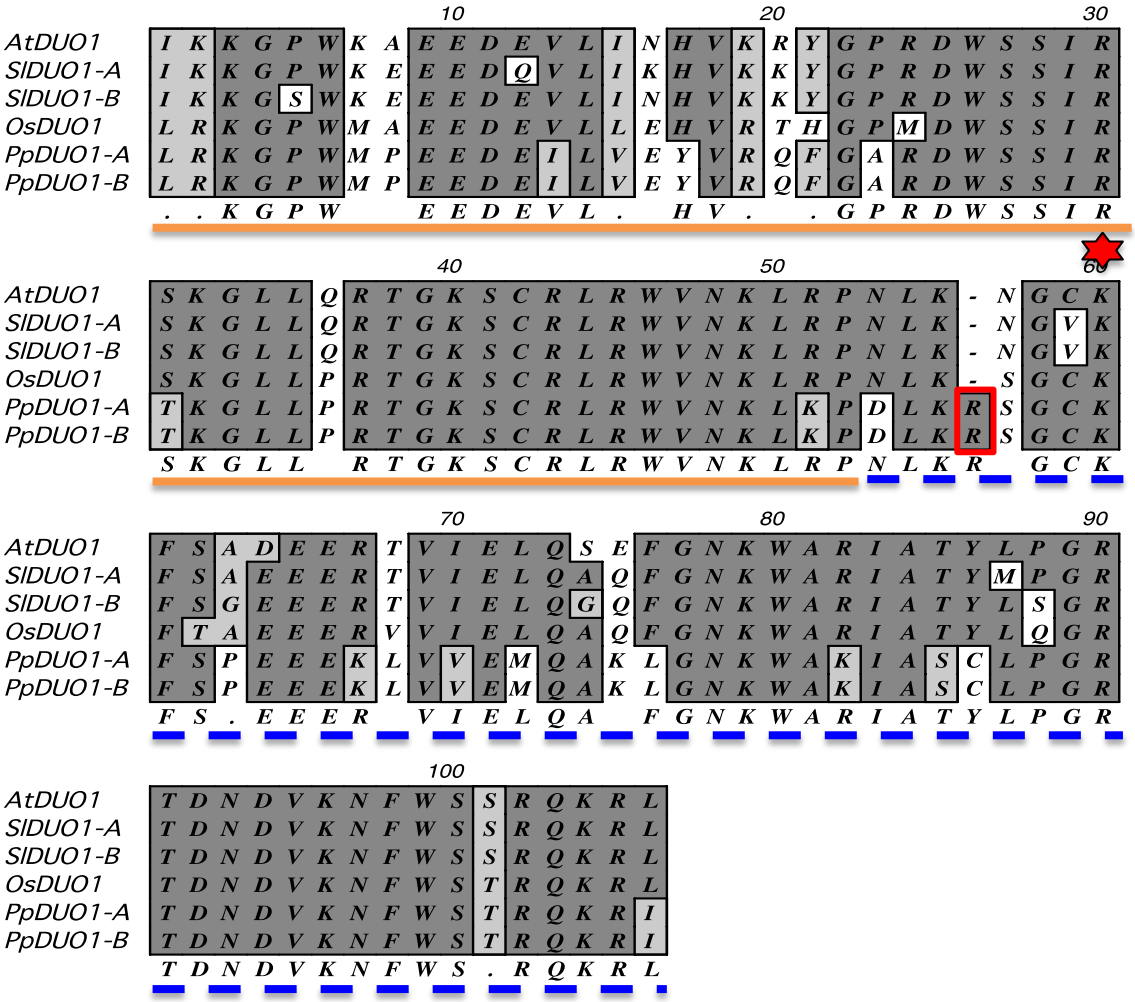


Figure 4.3: ClustalW alignment of the MYB domains of DUO1 in selected species. A typical R2R3 MYB repeat is about 106 amino acids long. All the sequence share high identities with each other. Signature lysine (K) residue which represented as a red star, is at the sixty-sixth position of the protein sequence in *Arabidopsis* DUO1. In Tomato DUO1-A & B, rice DUO1, and moss DUO1-A&B lysine is at the sixty-second (62nd), eightieth (80th), eighty-seventh (87th), seventy-second (72nd), and one hundred and ninety-seventh (197th) respectively. The two copies of DUO1 in moss have one extra amino acid Arginine (R) which is indicated in red rectangular before DUO1 (GCK) separately compare to the other species. The upper fragment is the R2 repeat which underlined with orange colour and the one is the R3 repeat that indicated blue dashed line.

4.5. Expression of *DUO1* in plants

4.5.1. Expression of *DUO1* homologs in *Solanum lycopersicum* (tomato)

In order to test for expression of *DUO1* in tomato, the first step was to isolate total RNA of different tissues, which are anther, leaf, and sepal-petal of the *S. lycopersicum 'Ailsa craig'* using a plant spectrum total RNA kit (Sigma). UV-Vis Spectrophotometer quantified the isolated total RNA samples. To test the quality 1µg was analysed and total RNA quality was confirmed by gel electrophoresis (Figure 4.4).

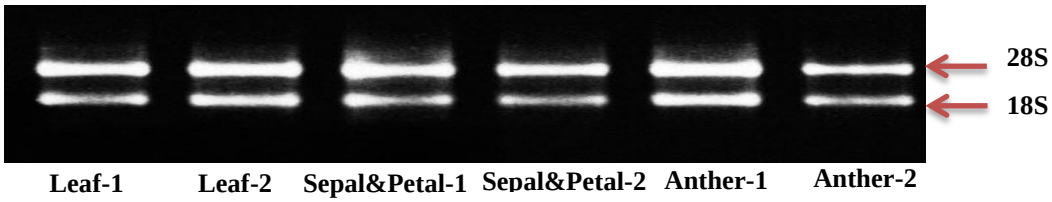


Figure 4.4: Agarose gel image of total RNA extracted from tomato tissues. Two samples total RNA was extracted from each of leaf, sepal-petal, and anther in *S. lycopersicum 'Ailsa craig'*. Two clear bands (28S and 18S) were visible without signs of degradation.

4.5.1.1. Reverse transcription polymerase chain reaction (RT-PCR) analysis of *DUO1* expression

For the expression analysis of *DUO1*, RT-PCR was performed. Equal amounts of total RNA (1 microgram) were used to produce single stranded cDNA. Gene Specific primer pairs, which are attB1SlDUO1-A F & attB2SlDUO1-A Rnostop and attB1SlDUO1-B F & attB2SlDUO1-B Rnostop, and Actin, were used for the RT-PCR. *SlDUO1-A* (Solyc01g090530.1) showed stronger expression than *SlDUO1-B* in anther, weak expression in leaf and no detectable expression in the sepal-petal. In contrast, another *DUO1* gene, *SlDUO1-B* (Solyc10g019260.1) showed any detectable expression like *SlDUO1-A* in leaf tissues. Actin primer pairs were used as a positive control to test cDNAs. It showed high expression in all tissues.

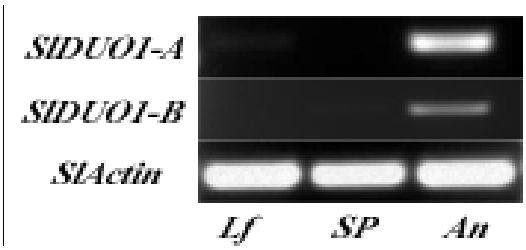


Figure 4.5: Analysis of the expression of *Solanum lycopersicum DUO1* homologs by RT-PCR. One tissue sample cDNA was used as a PCR template .Lf indicate leaf, SP; sepal-petal, and An; anther respectively. *SlDUO1-A* and *SlDUO1-B* showed clear anther-specific expression and appears from the signal to be the major *SlDUO1-A* gene expressed in anthers, however *SlDUO1-B* also showed evidence lower expression in anthers. Thus, both genes appear to be expressed, *SlDUO1-A* appears to be the major gene. *SlDUO1-A*, and *SlDUO1-B* were indicated that *SlDUO1-A&B* forward and reverse RT primer pairs. Actin primer was used positive control and it showed expression in all tissues.

4.5.1.2. Cloning and sequencing of the *Solanum lycopersicum SlDUO1A* and *SlDUO1B* genes

Employing the anther cDNA to gain access to the full-length fragment with Gateway two steps PCR for cloning DNA into pDONR vectors, *SlDUO1-A & B* primer pairs were used with attB1 and attB2 sites to try and predict the expected size in PCR-1 reaction. The correct band is excised from the gel, and the second PCR was undertaken with attB1 and attB2 adapter primers, which were the donor vector the researcher is cloning in. The predicted size band was pinpointed from the gel and the extracted *SlDUO1 A-B* fragments were cloned into the Gateway vector pDONR221, followed by Gateway recombination using Invitrogen's patented BP Clonase II plus. Afterwards, they transformed into competent *E. coli* cells. A colony screen was undertaken using generic primers flanking the cloning site to make sure insertion in the interested genes into the *E. coli* to highlight the entire sequence. Sequencing results showed that the complete sequence alignment was matched perfectly with predicted sequence.

4.5.2. Expression of DUO1 in *Oryza sativa* (Rice)

4.5.2.1. Tissue specific *DUO1* expression in *Oryza sativa*

Total RNA was extracted from various tissues, root, shoot, leaf, and different stages of

anther, from rice to test for expression DUO1.. These experiments were completed by Dabing Zhang (School of Life Science and Biotechnology, Shanghai Jiao Tong University). In order to, understand expression pattern of DUO1 in rice different tissues, LI Su-ling was performed RT-PCR. (College of fisheries and life, Shanghai Ocean University, Shanghai, China) According to RT-PCR result, DUO1 showed expression only mature pollen which is stage-12 (Figure 4.6)

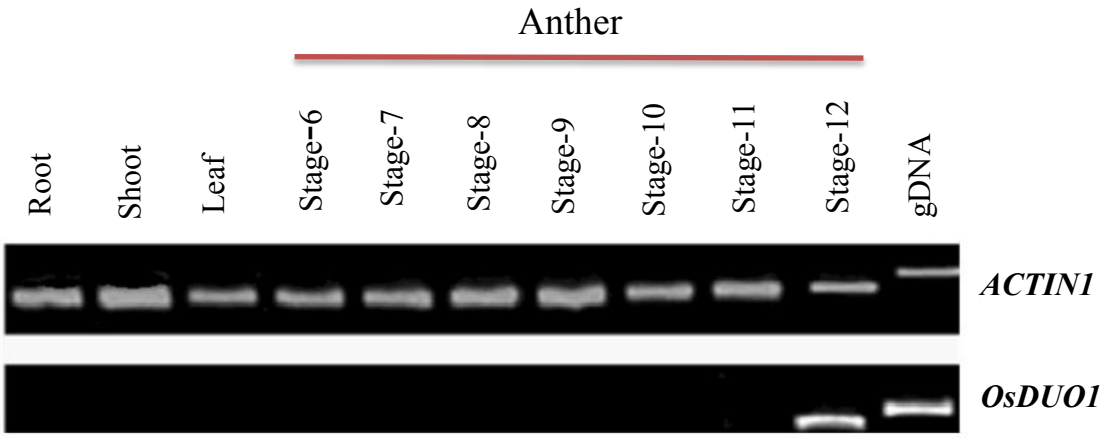


Figure 4.6: Expression of *OsDUO1* in various tissues. Root, shoot, leaf, stage1-6: microsporocyte development stage; stage7: microsporocyte stage; stage8: microsporocyte meiosis stage; stage9: early and middle microspore stage; stage10: late microspore stage; stage11: bicellular pollen stage; stage12: mature pollen stage; gDNA: rice genomic DNA. *ACTIN1* primer pairs were used as a positive control to test cDNAs. and showed uniform expression in all tissues. Adapted from Li *et al*. (2010).

4.5.2.2. Cloning and sequencing the *Oryza sativa DUO1*

Mature pollen stage cDNA was provided by Professor Dabing Zhang to get clone using Gateway two steps PCR with Velocity polymerase into pDONR 221 vectors. Cloning of the *OsDUO1* mature pollen was carried out by Eleanor Vesty (an MSc student in our lab). Than, they transformed into competent *E. coli* cells. A colony screen was then carried out with generic primers flanking the cloning site to confirm insertion our interested genes into the *E.coli*. Sequencing results showed that the complete sequence alignment was matched perfectly with predicted sequence.

4.5.3. Expression of *DUO1* in *Physcomitrella patens* (Moss)

4.5.3.1. Observation of reproductive parts in *Physcomitrella patens*

In order to test expression of DUO1 from moss, reproductive parts were observed for total RNA isolation. To observe reproductive parts under the microscope, first of all, cut away the apex of gametophore with gametangia from stem, and place the tissue into DAPI solution. Then incubated at 37 ^{o}C for 2 hours. Antheridia (male) and Archegonia (female) are formed together at the shoot apex of gametophores. They were covered with several young green parts, and it was necessary to remove the green parts for observation. Developmental processes at cellular level were observed by differential interference contrast microscope.

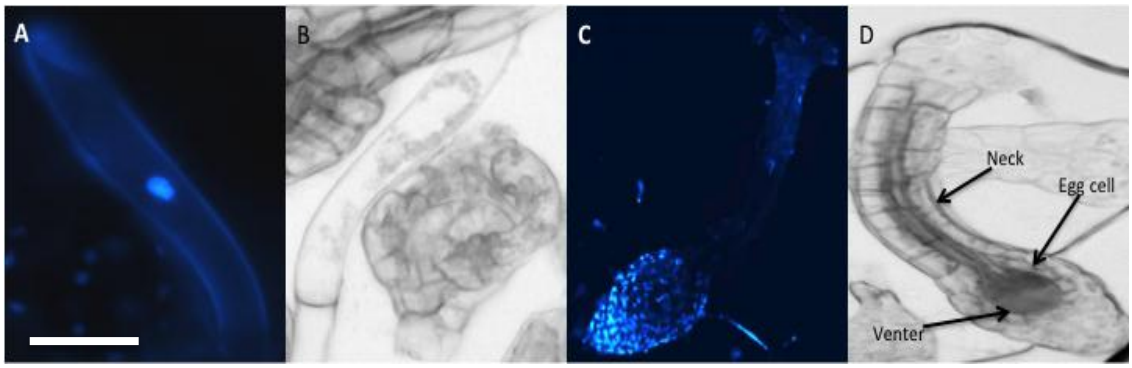


Figure 4.7: Observation of antheridia and archegonia in *Physcomitrella patens*. A: single celled antheridia initial show a single nucleus that is stained with DAPI, and then development of vacuole was observed using differential interference contrast microscopy (B). C-D: Each archegonium holds one egg (in a swollen section called the venter) and the sperm enter through the channel in the narrower, tubular section (or neck). Scale bar =10 μm

4.5.3.2. Tissue-specific *DUO1* expression in *Physcomitrella patens*

The first step was to collect different tissue parts, which are vegetative, sporophyte, and reproductive from *Physcomitrella patens*. After observation of the reproductive parts in moss, approximately 100 subcultures were checked and mixture antheridia and archegonia collected. Also moss spores were grown and ready to collect after two months. Only mature spores that seen brown colour were collected as sporophytic part

from subcultures. Leaf-like tissues were collected as a vegetative part (non-reproductive) tissue. Total RNA was extracted from collected tissues using a protocol plant spectrum total RNA kit (Sigma). UV-Vis Spectrophotometer quantified the isolated total RNA samples. To test the quality 1μg was analysed and total RNA quality was confirmed by gel electrophoresis.

4.5.3.3. RT-PCR analysis of *DUO1* expression

For the expression analysis of DUO1 in *Physcomitrella patens* vegetative-sporophytic and reproductive tissues, RT-PCR was performed. Equal amounts of total RNA (1 microgram) were used to produce single stranded cDNA with M-MLV reverse transcriptase which to make a cDNA copy of the mRNA. Gene Specific primer pairs, were used for the RT-PCR. Samples were taken after 35 of cycles (55 ^{o}C annealing) and analysed on agarose gel.

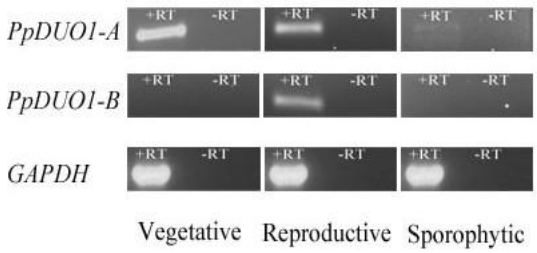


Figure 4.8: Expression of *Physcomitrella patens* tissues DUO1 homologues in RT-PCR. Reproductive part (male and female mixture) cDNA gave the expected size (414bp) for the PpDUO1-A RT primer pair). It also gave the expected cDNA size (439bp) for the PpDUO1-B RT primer pair. Vegetative part cDNA showed unexpected sizes (700 - 800bp) for the PpDUO1-A RT primer pair, but no signal for PpDUO1-B RT primer pair. Sporophytic part cDNA also showed similar profile size with Vegetative cDNA that maybe reflect mixed tissue in samples used for RNA extraction. GAPDH primer pairs were used as a positive control to test cDNAs. It showed high expression in all tissues.

4.5.3.4. Cloning and sequencing the *Physcomitrella patens* *PpDUO1-A* and *PpDUO1-B* genes

Using the reproductive cDNA to gain access to the full-length fragment with Gateway two steps PCR. The correct band is excised from the gel, and the second PCR was

undertaken with attB1 and attB2 adapter primers, which were the donor vector. The predicted size band was pinpointed from the gel and the extracted *PpDUO1 A-B* fragments were cloned into the Gateway vector pDONR221, followed by Gateway recombination using Invitrogen's patented BP Clonase II plus. Afterwards, they transformed into competent *E. coli* cells. A colony screen was undertaken using generic primers flanking the cloning site (M13F + M13R) to make sure insertion in the interested genes into the *E. coli* to highlight the entire sequence. Two colonies from *PpDUO1-A* and one colony from *PpDUO1-B* gene were sent for sequencing.

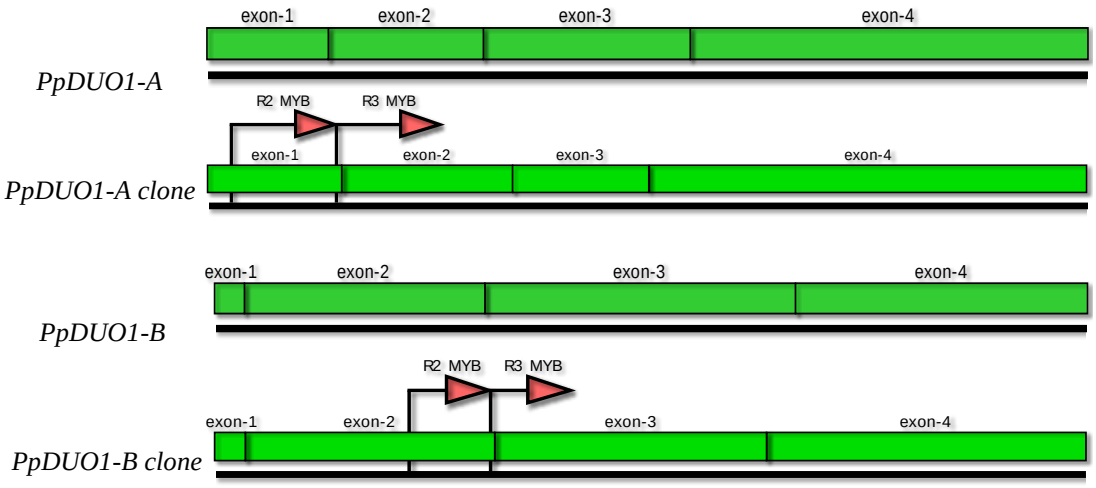


Figure 4.9: Gene Structures of the predicted and cloned sequences from *PpDUO1-A* and *PpDUO1-B*. *PpDUO1-A* colonies showed that exon-3 length was 140 bp shorter than the predicted sequence; other exons complete sequence alignment was perfectly matched with predicted sequence. *PpDUO1-B* colony showed; the first part of the gene that is first 60 bp was fully matched, than from 60bp to 92 bp was mismatched. From 92 to 840 bp which including R2R3 MYB domain was perfectly matched. Between the 840-1242bp were mismatched, and finally second part of the gene that is exon-4 was matched perfectly. R2R3 MYB domain that contain DUO1 gene was conserved in moss reproductive part (male and female mixture).

4.6. Activation of AtDUO1 targets transient expression assays in tobacco leaves.

Even though the results of the RT-PCR data show the *DUO1* expression potential of the selected species DUO1 homologs, this data by itself cannot pinpoint the minor disparities of the DUO1 variants and as a result, a quantitative approach was used in the form of a sensitive luciferase assay. Luciferase-based assays allow for a straightforward,

fast, and sensitive way of studying promoter transactivation (Koncz *et al.*, 1990) and have been effectively employed in the analysis of pollen gene promoters (Bate & Twell, 1998). Firefly luciferase is an enzyme, which is able to catalyze adenosine triphosphate (ATP)-dependent oxidation of its substrate luciferin to create light (Deluca & McElroy, 1978). *Renilla* luciferase is a distinct enzyme that also has the capability of catalyzing a luminescent reaction by employing O_2 and coelenterate luciferin (coelenterazine) (Matthews *et al.*, 1977). Both reporter genes cooperating in a single assay, which is commonly named a dual luciferase assay, allows for a powerful method capable of being controlled internally by normalizing the activity of firefly luciferase with that of *Renilla* luciferase (Sherf *et al.*, 1996). Combined with *Agrobacterium*-mediated transformation of tobacco leaf Sparkes *et al.* (2006), this type of system can facilitate a powerful transient assay, generating quantitative data within a two-day timeframe for minimal expense in comparison. As a result, there has been extensive investment in designing this style transient luciferase assay to be able to quantify the transactivation potential of the DUO1 variant proteins. Two target promoters were employed as the reporters in this assay, which were pMGH3 and pDAZ1 in *Arabidopsis*, tomato, and rice. In moss one target promoter that was pMGH3 was used. These two genes have been referred to with regards to the control of sperm cell differentiation (Borg *et al.*, 2011). Other germline specific genes (e.g. *GCS1* and *GEX2)* are commonly expressed more weakly and can also display expression in other plant tissues. As a result, it was agreed that pMGH3 and pDAZ1 were superior candidates for expression analysis. *MGH3* is normally expressed only in the male germline and plays a role in the differentiation of sperm cells.

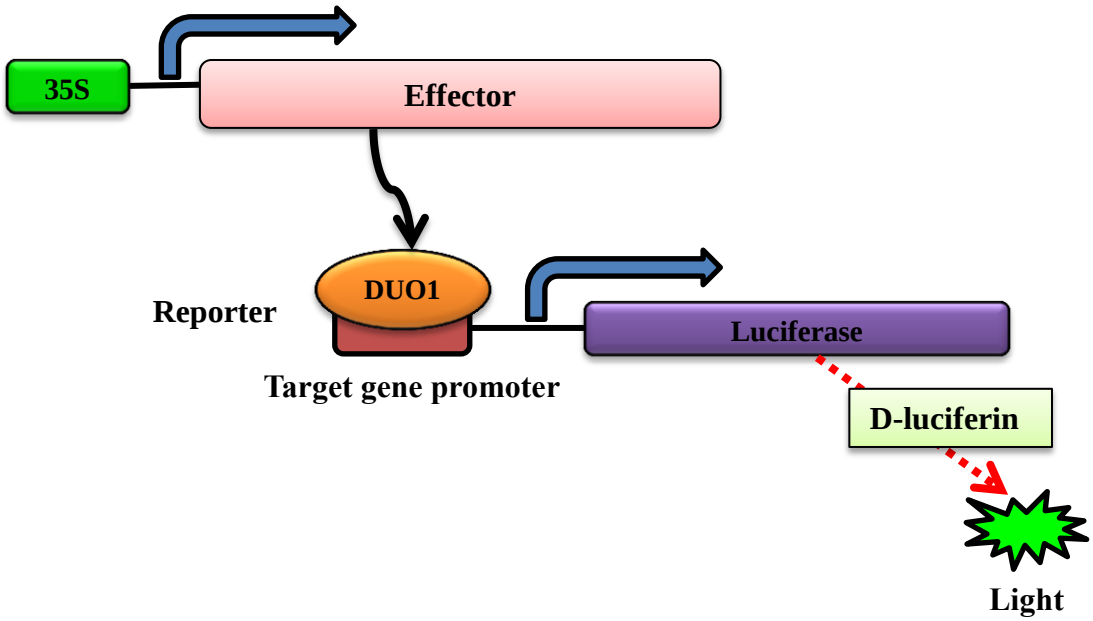


Figure 4.10: Method of luciferase production by action of two plasmids. Effector plasmid includes the species specific *DUO1*, controlled by a CaMV 35S promoter. The DUO1 protein created should then have the capability of binding to a target promoter in a reporter plasmid, in order to promote expression of luciferase. When ATP is present, O_2 and Mg, luciferase will oxidize D-luciferin to generate a quantifiable release of bioluminescence.

To be able to test transactivation of target genes, effector constructs are made up of the 35S promoter driving, which are *Physcomitrella patens* (Pro35S: PpDUO1-A, and Pro35S: PpDUO1-B), Oryza *sativa* (Pro35S:OsDUO1), *Solanum lycopersicum* (Pro35S: SlDUO1-A and Pro35S: SlDUO1-B) and (Pro35S: AtDUO1), which are produced in plant binary vectors using Gateway recombination DUO1 (Figure 4.11). The reporter vector were made up of ProMGH3: LUC (a canonical DUO1 target promoter), whereas the effector vectors were made up of the 35S promoter fused to the coding sequence of the genes to be tested. *Agrobacterium* strains with ProMGH3: LUC and ProDaz1: LUC were employed to infiltrate and transiently transform tobacco leaves with and without *Agrobacterium* strains with effector constructs. Every infiltration further included an *Agrobacterium* strain, which involved Pro35S: RenLUC as a control. These were examined 48 hours later for dual luciferase activity.

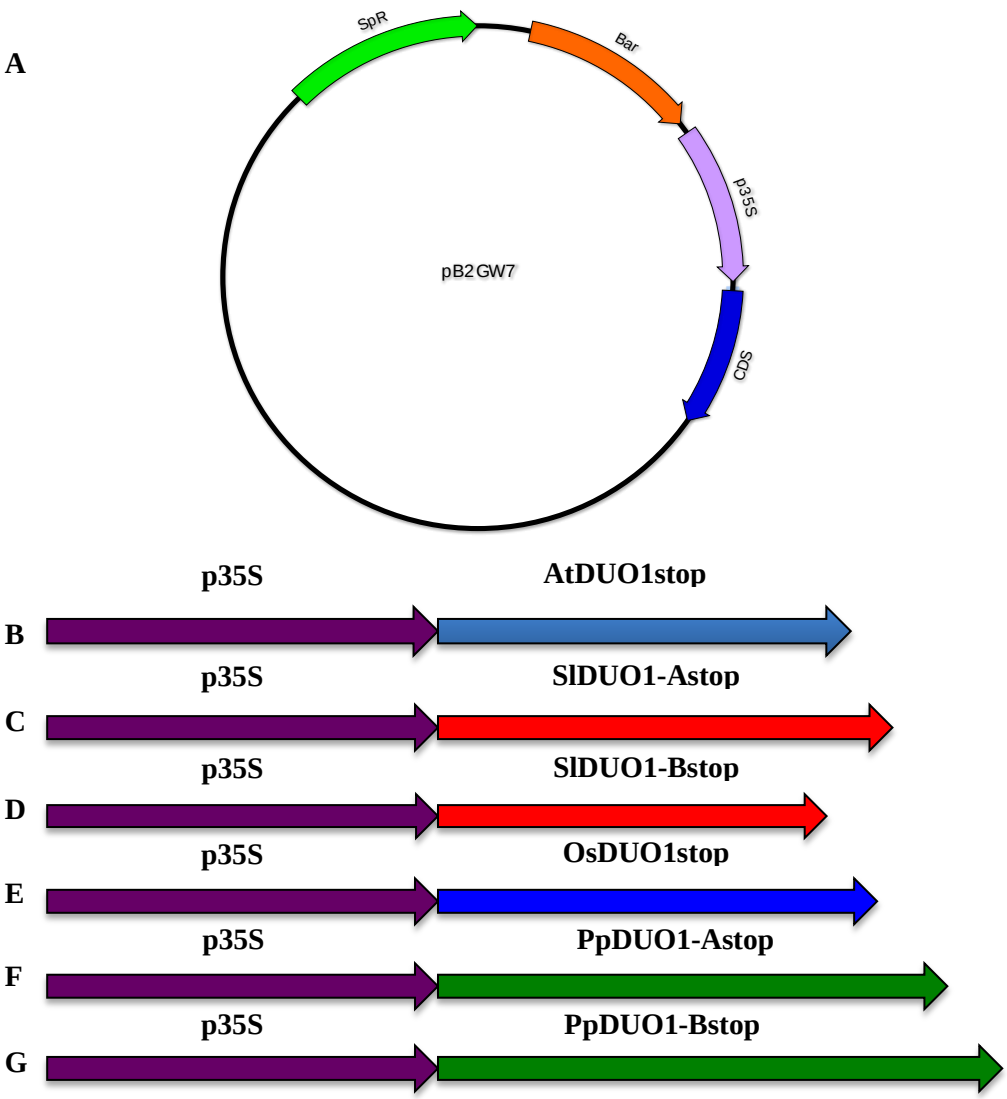


Figure 4.11: Structure of the species constructs map for transient luciferase assay in tobacco leaf. Circular map image (A) showed the structure of the 2-part construct which recombining with the destination vector pB2GW7. Binary vectors of the six components were labeled B to G. These corresponded to AtDUO1stop, SlDUO1-Astop, SlDUO1-Bstop, OsDUO1stop, PpDUO1-Astop, and PpDUO1-Bstop, respectively. The CaMV 35S promoter utilized in the transient luciferase assay promoted them. SpR and Bar are resistance genes (spectinomycin and Basta respectively).

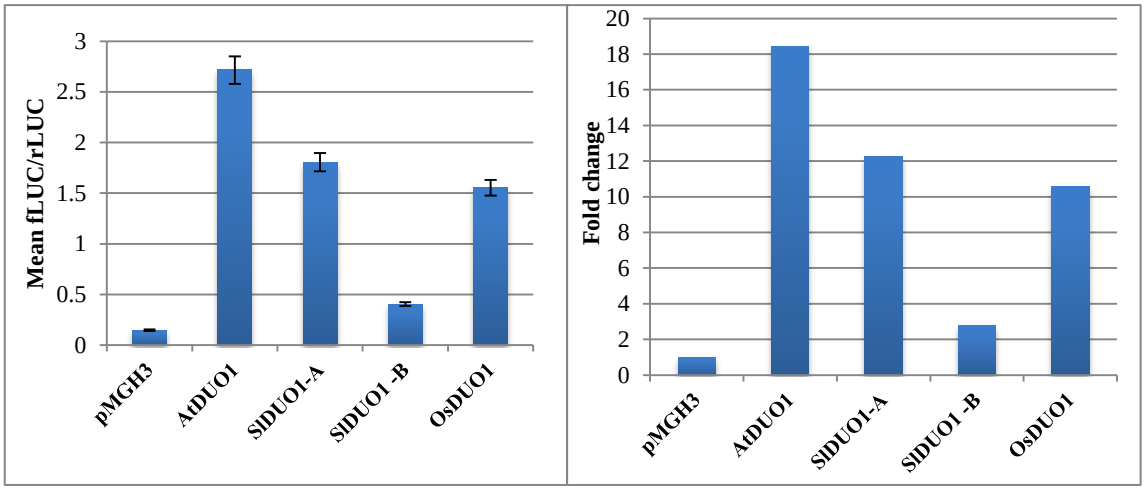


Figure 4.12: Bar graphs of the activation of DUO1 target promoter pMGH3 by DUO1 constructs. Mean firefly luciferase/renilla luciferase figures from the assay were duplicated on the same day to provide a more general value for every construct. AtDUO1 displayed the greatest amount of activation of pMGH3. *SlDUO1-A* displayed activation roughly 6 times higher than the *SlDUO1-B.SlDUO1-B* construct, which also displayed the lowest level of activation compared with the others. *OsDUO1* and *SlDUO1-A* constructs displayed similar levels of activation to pMGH3.

Table 4.1: Two-Paired T-test for the activation of DUO1 target promoter pMGH3 by DUO1 constructs. Statistical analysis of the transactivation of pMGH3 by DUO1 constructs was undertaken with the help of two-paired T-test that find out two values in same population. SlDUO1-B showed no significantly difference with pMGH3. Also, OsDUO1 showed no significantly difference with SlDUO1-A. *** P-value<0.2

Paired T-test	P-value	Significance
pMGH3-AtDUO1	0.000175022	***
pMGH3-OsDUO1	7.15913E-05	***
pMGH3-SlDUO1-A	0.005598182	***
pMGH3-SlDUO1-B	0.020756991	ns
AtDUO1-OsDUO1	0.031770642	***
AtDUO1-SlDUO1-A	0.165226317	***
AtDUO1-SlDUO1-B	0.000429009	***
OsDUO1-SlDUO1-A	0.478603871	ns
OsDUO1-SlDUO1-B	0.000520335	***
SlDUO1-A-SlDUO1-B	0.018022331	***

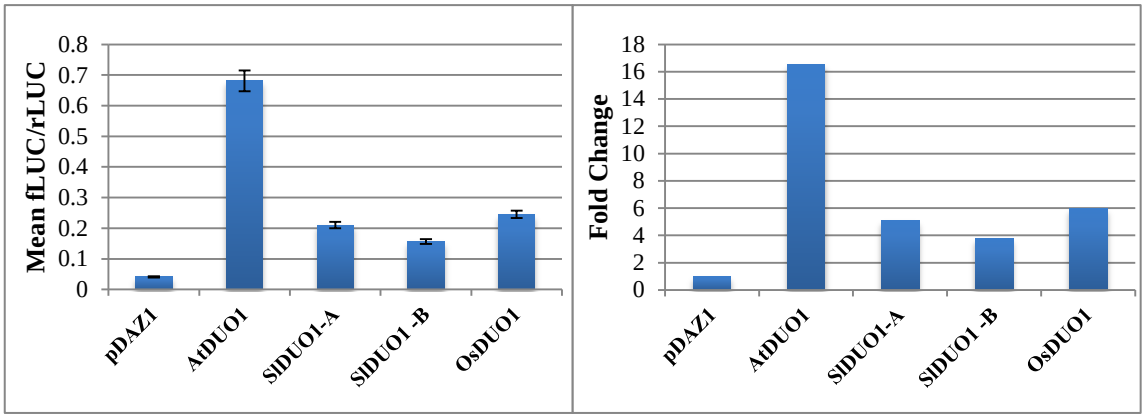


Figure 4.13: Bar graphs of the activation of DUO1 target promoter pDAZ1 by DUO1 constructs. Mean firefly luciferase/renilla luciferase figures from the examination were duplicated on the same day to provide a more general value for very construct. The result shows that at the present of AtDUO1, the promoter activation of pDAZ1 has sixteen folds increase. On the other hand, the greatest amount of activation of Arabidopsis was displayed by pMGH3. As before, the SlDUO1-B construct displayed the lowest level of activation. SlDUO1-A displayed a lower level of activation in pDAZ1, compared with pMGH3. The OsDUO1 construct displayed a greater higher transactivation level than the tomato constructs in pDAZ1.

Table 4.2: Two-Paired T-test for the activation of DUO1 target promoter pDAZ1 by DUO1 constructs. Statistical analysis of the transactivation of pDAZ1 by DUO1 constructs was undertaken with the help of two-paired T-test that find out two values in same population. Arabidopsis, tomato, and rice DUO1 constructs showed significantly difference value of the pDAZ1. However, the values of the SlDUO1-A and OsDUO1 showed no significantly difference each other. *** P-value<0.2

Paired T-test	P-value	Significance
pDAZ1-AtDUO1	9.50581E-06	***
pDAZ1-OsDUO1	0.000135965	***
pDAZ1-SlDUO1-A	0.000985889	***
pDAZ1-SlDUO1-B	3.69771E-05	***
AtDUO1-OsDUO1	0.000704045	***
AtDUO1-SlDUO1-A	0.000804948	***
AtDUO1-SlDUO1-B	5.91439E-05	***
OsDUO1-SlDUO1-A	0.233021622	ns
OsDUO1-SlDUO1-B	0.015731425	*
SlDUO1-A-SlDUO1-B	0.079714232	***

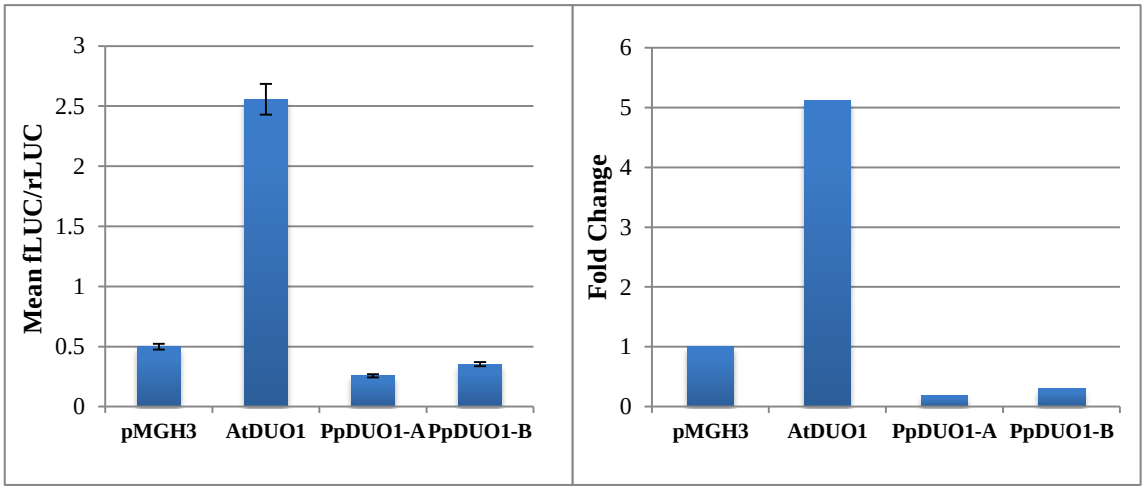


Figure 4.14: Activation of DUO1 target promoter pMGH3 *by Physcomitrella patens* DUO1 homologs constructs. AtDUO1 representing the positive control could activate its target. Despite this, the luminescence levels of two PpDUO1s were not considerably different from the negative control. The findings also displayed that the presence of AtDUO1, the promoter activation of MGH3, increased by five times.

Table 4.3: Two-Paired T-test for the activation of DUO1 target promoter pMGH3 by DUO1 constructs. Statistical analysis of the transactivation of pMGH3 by DUO1 constructs was tested with the help of two-paired T-test that find out two values in same population. AtDUO1 showed no significantly difference with pMGH3 and PpDUO1-A&B. Moss DUO1-A and B showed no significantly difference with pMGh3 and also each other. *** P-value<0.00.1, ns: no significant

Paired T-test	P-value	Significance
pMGH3 - AtDUO1	0.00042	***
pMGH3-PpDUO1-A	0.16365	ns
pMGH3-PpDUO1-B	0.29500	ns
AtDUO1-PpDUO1-A	0.00001	***
AtDUO1-PpDUO1-B	0.00004	***
PpDUO1-A-PpDUO1-B	0.11476	ns

4.7. In planta complementation analysis of DUO1 variants

By identifying *DUO1* homologs has given researcher the chance to examine plant reproduction in an evolutionary vein. The eudicot disparity is a key happening in the evolution of angiosperms. *Oryza sativa* (rice) and *A. thaliana* can be employed to look at the difference of this split and through this explore 150 million years of evolution. Despite the fact that the lineages of the Brassicaceae (*Arabidopsis)* and Solanaceae, (*S. lycopersicum*) families have approximately 112 million years ago between them, what their corresponding genomes have been undergoing since then can be seen in the order and the number of the genes that they have in common. By comparing the genomes of *Arabidopsis* and tomato a picture of how plant genomes have evolved since the radiation of dicotyledonous plants can be drawn, which can subsequently cover the key crops that come under the *Arabidopsis*/tomato category. Mosses, such as *Physcomitrella patens* are a very successful and common group, and their basal position in the land plant evolutionary tree puts them in a position to provide significant evolutionary developmental data. As a result, it was possible to look into the complementation of these homologues and highlight any conservation of function throughout these evolutionary divergent plants. With *in vitro* protein expression and *in planta* complementation assays, it was discovered that the function of DUO1 has been maintained from a common ancestor of the monocot and dicot lineages.

4.7.1. Production of the selected species DUO1 constructs

In order to evaluate the complementation potential of selected species which are *A. thaliana, S. lycopersicum, O. sativa and P. patens,* DUO1 constructs were generated in which the *Arabidopsis* DUO1 promoter was used to drive expression of an inserted gene CDS (coding DNA sequence) with mCherry (transgene) fusion protein using 3-part gateway cloning system by Invitrogen (figure 4.15). The reason to attach mCherry with DUO1 construct is because transcription of DUO1 could be visualized with Red fluorescence protein under UV light. *Arabidopsis* and rice constructs were introduced into *duo1-1/+* plants; tomato and moss constructs were introduced into *duo1-4/+* using floral dipping method.

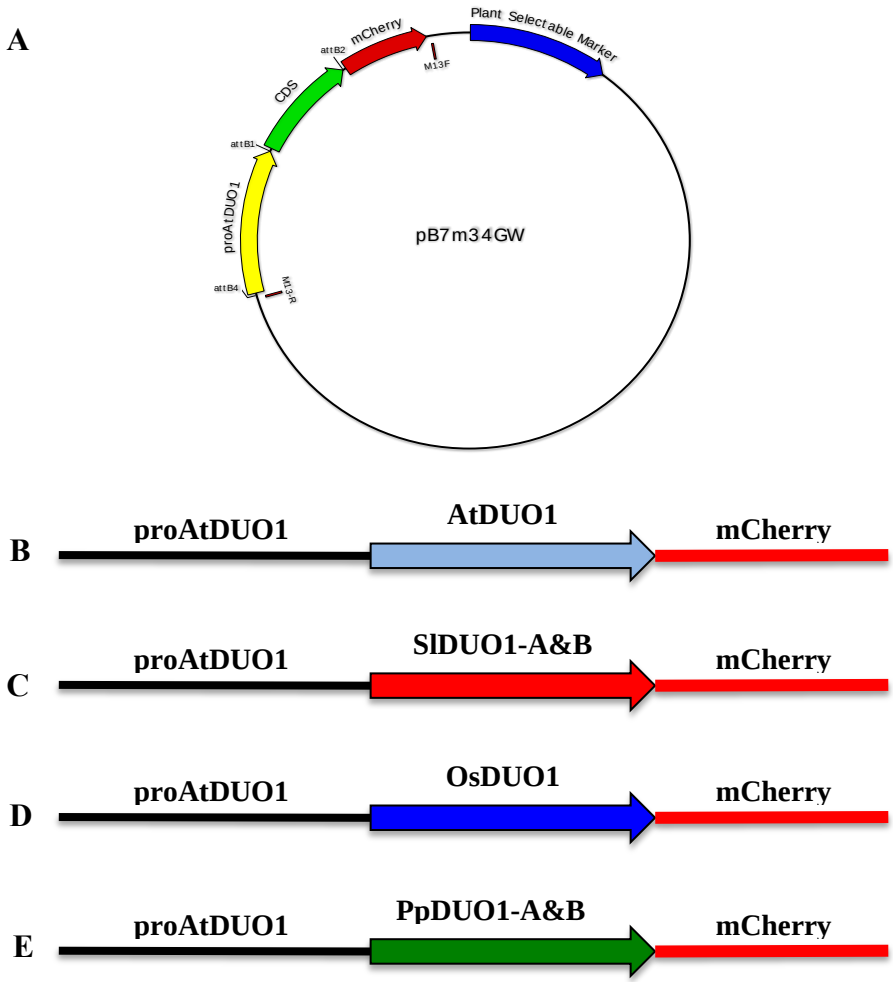


Figure 4.15: Structure of PromDUO1:DUO1-mCherry fusion constructs from different species in Gataway T-DNA destination vectors. A circular plasmid map (A) showing the structure of the 3-part constructs of the DUO1 orthologs and homologs. These constructs vectors structure of the six components were labeled B to E. These corresponded to AtDUO1-Anostop-mCherry, SlDUO1-A&Bnostop-mCherry, OsDUO1Bnostop-mCherry, and PpDUO1-A&Bnostop-mCherry, respectively. "nostop" means this construct contains a translational fusion to the reporter gene.

To be able to analyse plants phenotypes, they supposed to be able accomplish transformation. Than successfully transformed plants T1 seeds were sown and watered with Basta (an herbicide, contact active component glufosinate that is made by selectable marker in the pB7m34GW destination vector). Following this, the position and intensity of DNA in pollen grain staining with DAPI is highlighted, so that by establishing these characteristics, they can be evaluated with regards to gene expression level in pollen. Half of the pollen population in a *duo1* mutant plant is made up of wild

type tricellular pollen, whereas the other mutant half of the population is bicellular. Restored mitotic division in *duo1* pollen grains generate tricellular pollen and can potentially show rescue of the *duo1* mutation.

4.7.2. Complementation potential of the DUO1 homologs in tomato

The T1 lines were primarily screened from lines to pinpoint representative duo1 lines with a unique insertion for the transgene and a comparative mRFP signal which showed ~50% mCherry. With regards to the lines examined, the frequency of duo1 pollen grains was established by scoring the number of bicellular and tricellular pollen grains by DAPI staining. Twenty-four specific plants of every construct were examined in isolation. In the cases where SlDUO1-A and B could not rescue the *duo1* phenotype, a 1:1 WT: *duo1* ratio would be examined. Despite this, the range of data for all *duo1* plants was extensive and most plants came under the normal range of % *duo1*, which identifies full rescue, at roughly 25% (figure 4.16).

From this first screening, specific lines were chosen for future research, especially those displaying ~25% *duo1* and those appearing as tricellular pollen. The number of loci was established by the % mCherry, with single locus lines displaying ~50% mCherry. Summing up the amount of pollen grains which are bicellular and tricellular then allowed a thorough analysis.

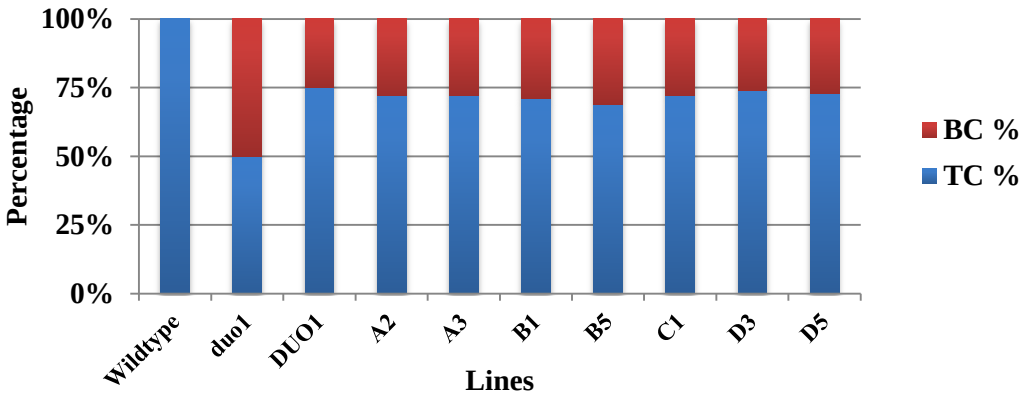


Figure 4.16: Complementation potential by SlDUO1-A protein

A bar chart is used to depict the complementation potential of the *SlDUO1-A* construct to rescue the cell cycle defect in the *duo1* mutant. Separate summations of bi- and tricellularity were undertaken with DAPI fluorescence. The examined Pollen from a *duo1-4*/+ plant without the transgene had ~50% tricellular pollen, the wild type plant had 100% and predicted full rescued DUO1 pollen had ~75% tricellular pollen. Seven plants were discovered to have a single locus and expression of *SlDUO1-A* transgenes also raised the percentage of wild type pollen to ~75%(ProDUO1:SlDUO1-A-mCherry), A2 plant showed 72% tricellular pollen, A3,B1,B5,C1,D3, and D5 were showed 72%, 71%,69%, 72%,74%, and 73% respectively. Thus expression of the tomato DUO1-A orthologue is able to complement the mitotic *duo1* defect and demonstrates conservation of function in *Arabidopsis*. BC: bicellular pollen, TC; tricellular pollen.

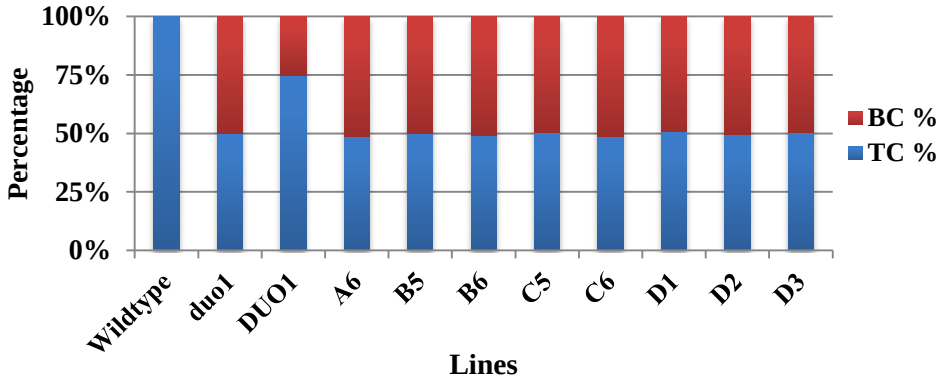


Figure 4.17: Complementation potential by SlDUO1-B protein. A bar chart represented to describe the complementation potential of the *SlDUO1-B* construct to rescue the cell cycle defect in the *duo1* mutant. Eight individual plants which are single locus lines showing ~50% mCherry, have been analysed .All plants showed approximately ~50% tricellular pollen that means expression of the tomato DUO1-B orthologue is not able to complement the mitotic *duo1* defect and demonstrates conservation of function in *Arabidopsis*. BC: bicellular pollen, TC: tricellular pollen.

In order to confirm the complementation potential of SlDUO1-A lines, the three higher percentages of tricellular pollen plants, SlDUO1-A C1, D3, and D5 plants, were selected to two more total pollen population counts to confirm reproducibly of the lines.

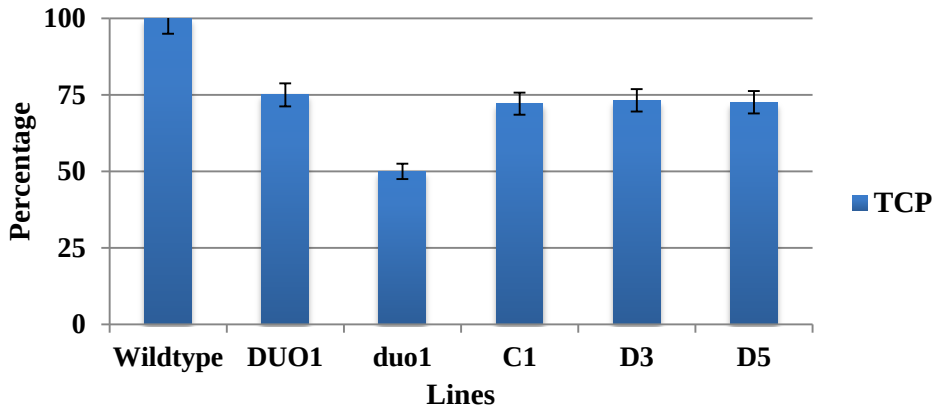


Figure 4.18: Complementation potential of DUO1 protein in selected SlDUO1-A plants. Overall three counts of the mean pollen population showed that *duo1-4/+* plant without the transgene had ~50% tricellular pollen, wild type plant had 100%. *SlDUO1-A* C1 showed 72%, D3, and D5 were showed 73% and 72.5% tricellular pollen. Expression of the tomato DUO1-A orthologous is able to complement the mitotic *duo1* defect confirmed two more times with counts. Error bars represent the standard error of the mean for the individuals analyzed. TCP: tricellular pollen.

4.7.3. Complementation potential of the DUO1 orthologs in rice

In order to assess the complementation potential of OsDUO1, two constructs were generated in which the *Arabidopsis* DUO1 promoter was used to drive expression of an OsDUO1-mCherry fusion protein (ProDUO1:OsDUO1-mCherry) as well as OsDUO1 protein alone (ProDUO1:OsDUO1). As a control, an additional mCherry fusion construct was generated in the same context with *Arabidopsis* DUO1 (ProDUO1:AtDUO1-mCherry). These three constructs were introduced into *duo1-1/+* plants and primary transformants (T1) screened for complementation of the *duo1* phenotype. Constructs were generated and observed initial T1 plants by Elenor Vesty. Twenty-four specific plants of every construct were examined in isolation. In the cases where If OsDUO1 could not rescue the *duo1* phenotype, a 1:1 WT: *duo1* ratio would be examined. However, the range of data within all *duo1* plants was quite large the majority of plants normal range of % *duo1* that represents full rescue, approximately

25% apart from ProDUO1:OsDUO1-mCherry constructs which showed 70% tricellular pollen.

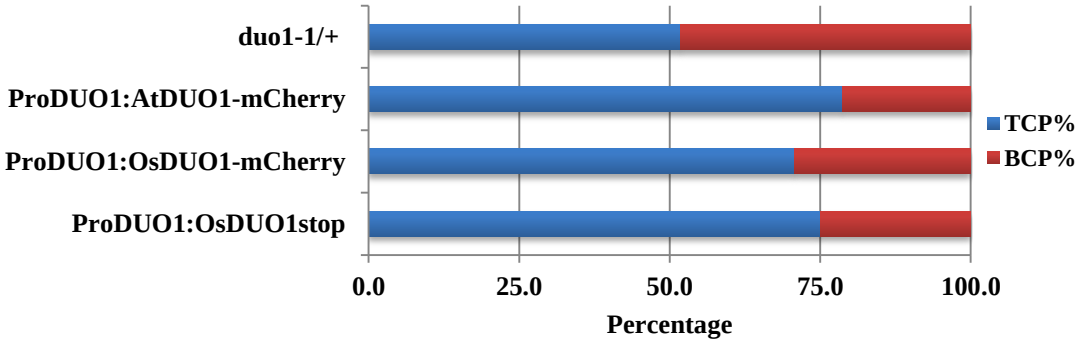


Figure 4.19: Complementation potential of the DUO1 protein in rice and *Arabidopsis*. Presented is the percentage tricellular pollen observed among the T1 lines analysed Pollen from a *duo1-1/+* plant without the transgene had ~50% tricellular pollen. In lines expressing the control construct ProDUO1: AtDUO1-mCherry, the number of tricellular pollen increased to ~79%. Similarly, expression of both OsDUO1 transgenes also increased the percentage of wild type pollen to ~70% ProDUO1:OsDUO1-mCherry, and 75% ProDUO1:OsDUO1stop. Thus expression of the rice DUO1 orthologue is able to complement the mitotic *duo1* defect and demonstrates conservation of function in *Arabidopsis*. TCP: tricellular pollen, BCP: bicellular pollen.

Two lines, which contained ProDUO1:OsDUO1-mCherry construct, B6 and C3 were harvested as a T2 generation. In order to confirm complementation potential of the construct and also to identify if the *duo1* mutant pollen grains rescued with the DUO1 variants could also fertilize and transmit genetic information to the resulting offspring those lines were sown and phenotypically and genotypically confirmed. Phenotypic observation results showed that ProDUO1:OsDUO1-mCherry construct was able to increase the percentage of tricellular pollen to ~75% in contrast to previous experiment result.

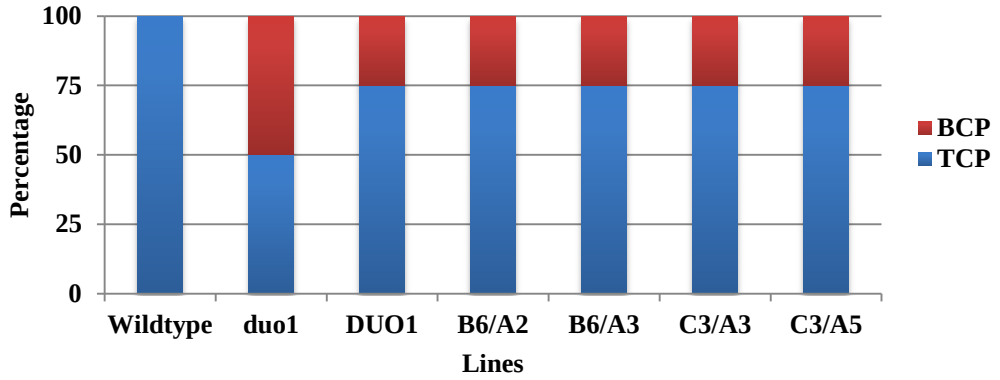


Figure 4.20: Complementation potential of DUO1 protein in rice T2 generation. Two plants from each T2 lines, B6 and C3, showed full rescue (~75% tricellular pollen).

In order to confirm *duo1* genotypically, B6 and C3 lines genomic DNA from leaf tissues were extracted using CTAB method. Than PCR was performed using dCAPS (Derived Cleaved Amplified Polymorphic Sequences) primer pairs, which generates a restriction site for the SspI enzyme in *duo1-1* plants. PCR products were digested with SspI-HF (New England Biolabs) at 37 ^{o}C for 1.5 hours (figure 4.21).

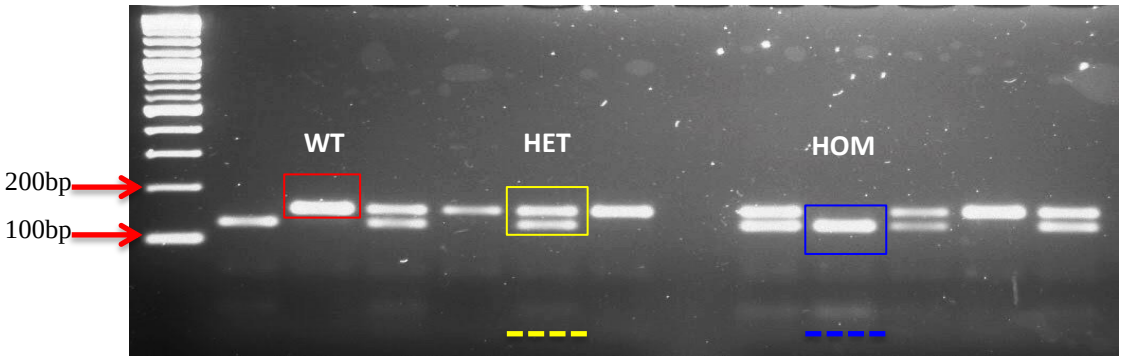


Figure 4.21: Identification of homozygous *duo1-1* plants expressing *OsDUO1*.
Agarose gel image representing of the restriction digested PCR products of rice DUO1 T2 generation . When PCR products were digested with SspI-HF restriction enzyme WT plants would produce one fragment of 150bp, which is indicated in the red rectangular shape, heterozygous plants containing duo1 would produce a pattern of three fragments, which are 150bp, 130bp and 20bp, indicated in the yellow rectangular shape and dashed line. Homozygous plants for *duo1-1* produces two fragments that are 130bp and 20bp, indicated in the blue rectangular shape and dashed line. Smaller fragments are difficult to see in most cases but presence of three fragments is indicative of *duo1* genotype. All PCR products were analysed by agarose gel electrophoresis (4%). WT: wildtype, HET: heterozygous, and HOM: homozygous

Heterozygous and homozygous *duo1-1* plants were selected to phenotype screen by counting tricellular and bicellular pollen grains and transgene. Finally, all lines were confirmed genotypically and phenotypically. Selected plants were crossed with *msI* male sterile plant.

4.7.4. Complementation potential of moss DUO1 orthologs.

BASTA selection of the PpDUO1-A and PpDUO1-B transformants produced a lower frequency of plants than selection of the rice DUO1 and tomato DUO1 (SlDUO1-A and B T1) transformants. The first selection of the transformants (out of 1000 seeds) plants produced no resistant plants. When all seeds were sown again, to get successful transformants. PpDUO1-B transformants showed higher frequency than PpDUO1-A. Twenty-four plants of each construct were analysed. Initial T1 screenings were observed approximately half of the plants duo1 from each line. Than, The amount of loci was established by the % mCherry, with single locus lines displaying ~50% mCherry. Nevertheless, none of the plants showed mCherry expression in *PpDUO1-A* line. Only three plants were observed mCherry expression in tricellular and bicellular pollen population from PpDUO1-B line. If the mutant phenotype was to be rescued, the PpDUO1-A construct had to be expressed. The absence of mCherry, in both WT and *duo1* pollen grains, suggests that it was not expressed (figure 4.22-4.24).

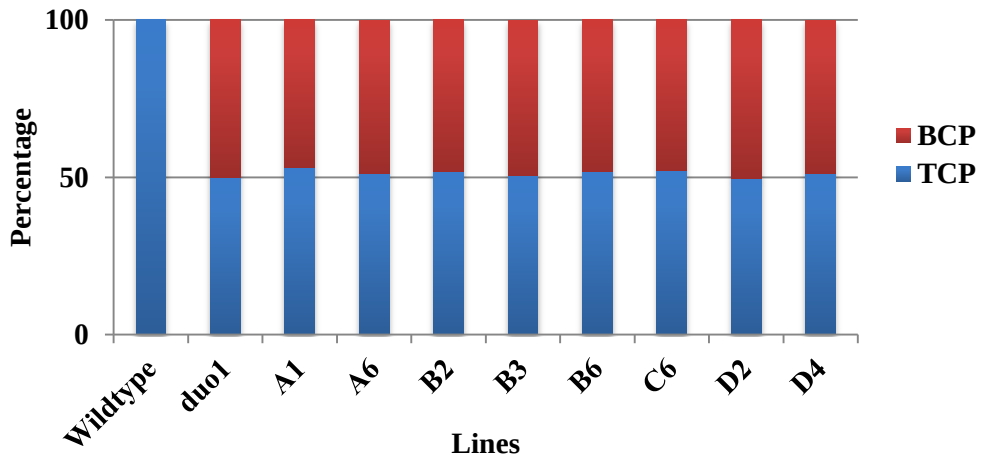


Figure 4.22: Complementation potential by PpDUO1-A. A bar chart is used to illustrate the complementation potential of the PpDUO1-A construct to rescue the cell division defect in the *duo1* mutant. Eight plants were analyzed phenotypically and all of them showed approximately ~50% tricellular pollen. Thus, expression of the moss DUO1-A ortholog is not able to complement the mitotic *duo1* defect indicating lack of conservation of function in *Arabidopsis*. BCP: bicellular pollen, TCP: tricellular pollen.

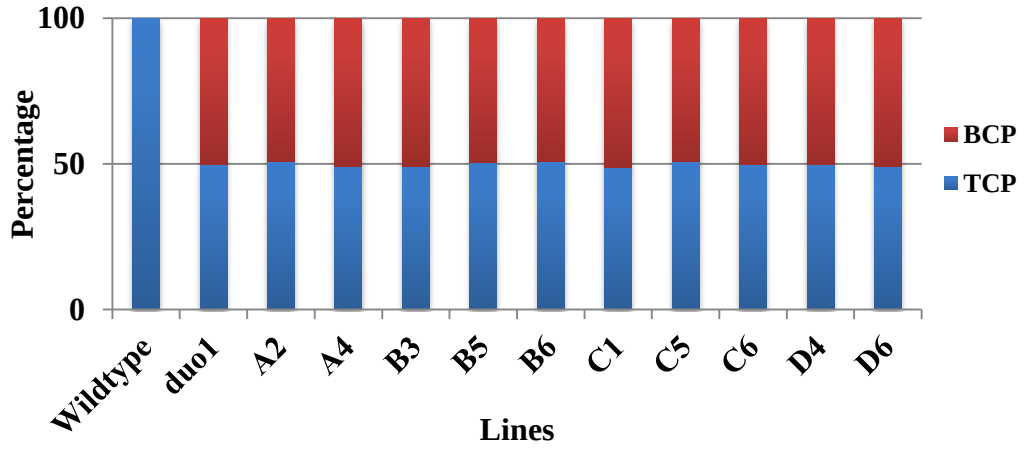


Figure 4.23: Complementation potential by PpDUO1-B. A bar graph is used to demonstrate the complementation potential of the PpDUO1-B construct to rescue the cell division defect in the *duo1* mutant. Ten plants were analyzed phenotypically and all showed approximately ~50% tricellular pollen. Thus indicates that expression of the moss DUO1-A ortholog is not able to complement the mitotic *duo1* defect and a lack of conservation of function in *Arabidopsis*. In contrast to the PpDUO1-A line, three PpDUO1-B plants, which are B5, C6, and D4 showed mCherry transgene expression . BCP: bicellular pollen, TCP: tricellular pollen.

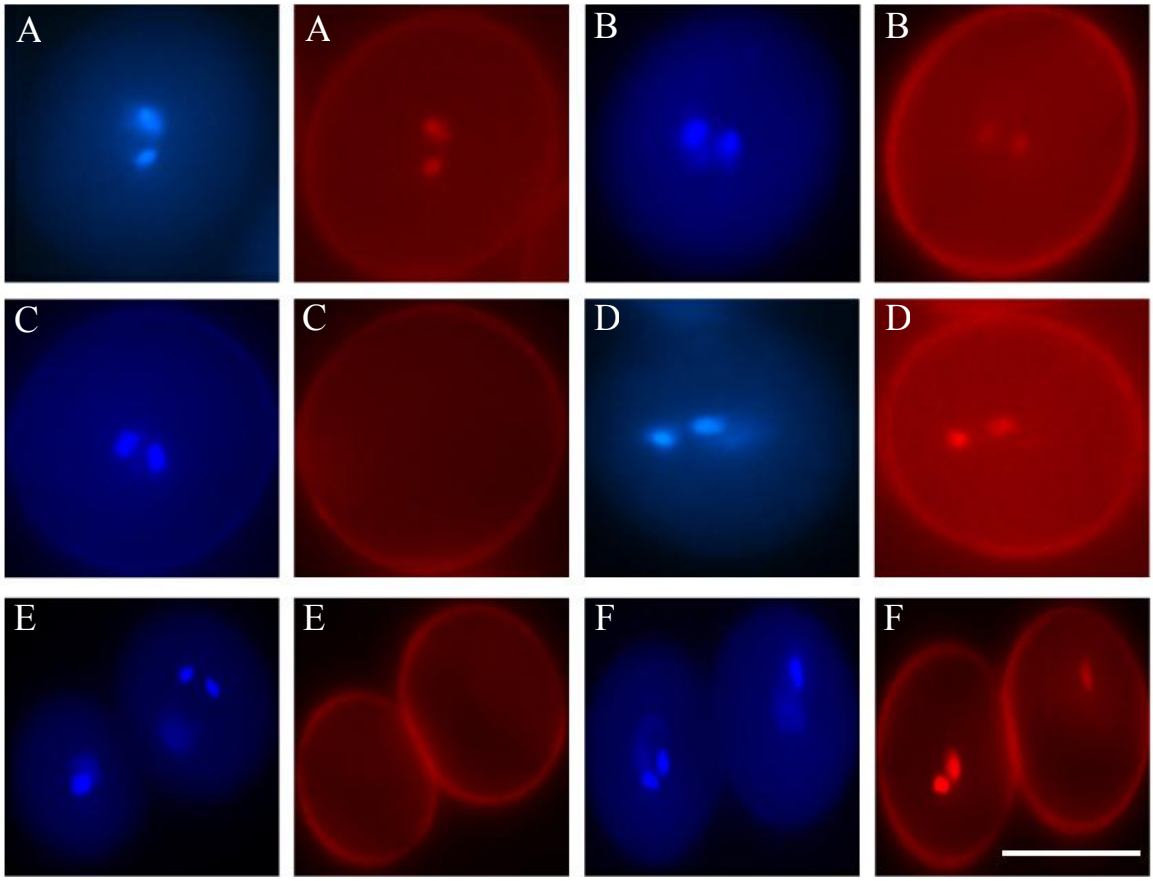


Figure 4.24: Images of DUO1 mCherry expression of T1 lines from selected species. Images were shown with DAPI and RFP for each construct from A to F that are ProDUO1:AtDUO1-A-mCherry,ProDUO1:SlDUO1-A-mCherry,ProDUO1:SlDUO1-B-mCherry,ProDUO1:OsDUO1B-mCherry ProDUO1:PpDUO1-A-mCherry, and ProDUO1:PpDUO1-B-mCherry respectively. Mature pollen from T1 lines harboring these constructs were stained with DAPI analyzed under RFP fluorescence. Tomato DUO1-A construct (B) showed weak transgene expression conversely *Arabidopsis* (A) and rice (D) DUO1 constructs. There is no expression in tomato DUO1-B (C) with moss DUO1-A (E). Some plants showed mCherry expression in PpDUO1-B line (F). Scale bar represents 10μm

Statistical analysis of the cell cycle rescue was carried out with the help of chi-square analyses, which evaluated differences in the examined ratio of wild type to duo1 pollen grains from a 3:1 ratio (a fully-rescued single locus line) and a 1:1 ratio (a non-rescued line). Related chi-square evaluations were undertaken to evaluate the number of *duo1* rescuing by testing deviations in the detected amount of tricellular pollen to bicellular pollen grains (Table 4.4). In line with predictions, the pollen classes observed in lines rescued with native DUO1 protein displayed no deviation from a fully rescued line (3:1)

but were quite different to a non-rescued line (1:1) for cell cycle rescue. As estimated, the pollen types observed in lines rescued with *AtDUO1*, *SlDUO1-A*, and *OsDUO1* protein showed no difference from a fully rescued line (3:1) but were significantly different from a non-rescued line (1:1) for both cell cycle rescue (Table 4.4). Conversely, SlDUO1-B, PpDUO1-A&B lines showed there is no significantly different from a non-rescued line (1:1).

Table 4.4: Cell cycle rescue of complementing DUO1 in species

						Based on a 3:1 ratio		Based on a 1:1 ratio	
Rescuing transgene	**Line**	**Total**	**TCP**	**BCP**	**TCP : BCP**	**χ^2 value**	**Significance**	**χ^2 value**	**Significance**
pDUO1-AtDUO1::Cherry	4	581	438	143	3.06:1	0.046	ns	149.785	***
pDUO1-SlDUO1-A::Cherry	8	1012	732	280	2.6:1	3.842	ns	201.881	***
pDUO1-SlDUO1-B::Cherry	8	946	465	471	0.99:1	320.051	***	0.038	ns
pDUO1-OsDUO1::Cherry	4	519	389	130	2.99:1	0.001	ns		***
pDUO1-PpDUO1-A::Cherry	8	1006	518	488	1.06:1	296.526	***	0.895	ns
pDUO1-PpDUO1-B::Cherry	10	1229	613	616	0.99:1	413.676	***	0.007	ns

4.8. Transmission efficiency of *duo1* pollen rescued with the DUO1 variants

The experiments with transactivation and complementation of DUO1 described above can act a source of extensive knowledge about DUO1 function. This biological importance, as a result, is based on the production of twin sperm cells, which can successfully fertilize the female gametes and assist the creation of the next generation. Subsequently, a final experiment was undertaken to try and establish if the *duo1* mutant pollen grains rescued with the DUO1 variants could also fertilize and transmit genetic information to the resulting offspring.

If the choice of lines satisfies the research, the transmission capacity of *duo1* pollen taken with the DUO1 variants was examined. Coming from the segregating population of every line, a *duo1* mutant that was heterozygous for the transgene was crossed as a male onto ms1 pistils. Under normal circumstances, the *duo1* mutation stops the pollen from going through fertilisation and as a result can only be maintained as a

heterozygote. If a single copy transgene can completely rescue the duo1 mutation, then half of the *duo1* pollen will include the transgene and thus have the ability to successfully fertilise and transmit. Because of this, *duo1* pollen without the transgene would not be able to transmit if the wild type pollen (25% with and 25% without the transgene) and the other 25% duo1 pollen containing the transgene were transmitting. As a result, the next generation would supposedly expect a 2:1 ratio of wild type plants (derived from the 50% wild type pollen) and duo1 plants (derived from the 25% rescued duo1 pollen).

F1 seeds were harvested from 2 lines from *Arabidopsis*, 3 lines from tomato, and 2 lines from rice. Afterwards, 200 F1seeds were sown from *Arabidopsis* and rice, more than 500 F1 seeds from tomato on Phosphinothricin (PPT) (10 mg/ml) antibiotic selection plates with wild type negative control.

Statistical analysis of the male transmission was undertaken with the help of chi-square analyses, which evaluated differences in the examined ratio of resistant to sensitive plants from a 2:1 ratio (a fully-transmitted line) and a 1:1 ratio (a non-transmitted line). Related chi-square evaluations were undertaken to evaluate the number of sensitive by testing deviations in the counted number of resistant to sensitive plants (Table 4.5).

Table 4.5: Transmission efficiency of DUO1 in different species. As expected, the resistant and sensitive plants were observed in lines fully transmitted with AtDUO1, SlDUO1-A, and OsDUO1 transmission efficiency showed no difference from a fully transmitted line (2:1) but were significantly different from a non-transmitted line (1:1) for three lines. (Table 2) Tomato and rice DUO1 showed approximately similar transmission ratio.

						Based on a 2:1 ratio		Based on a 1:1 ratio	
Rescuing transgene	Line	Total	PPT^R	PPT^S	PPT^R:PPT^S	χ^2 value	Significance	χ^2 value	Significance
pDUO1-AtDUO1::Cherry	2	200	132	65	2.0:1.0	0.010	ns	22.787	***
pDUO1-SlDUO1-A::Cherry	3	525	335	174	1.9:1	0.166	ns	50.925	***
pDUO1-OsDUO1::Cherry	2	200	118	62	1.9:1	0.100	ns	17.422	***

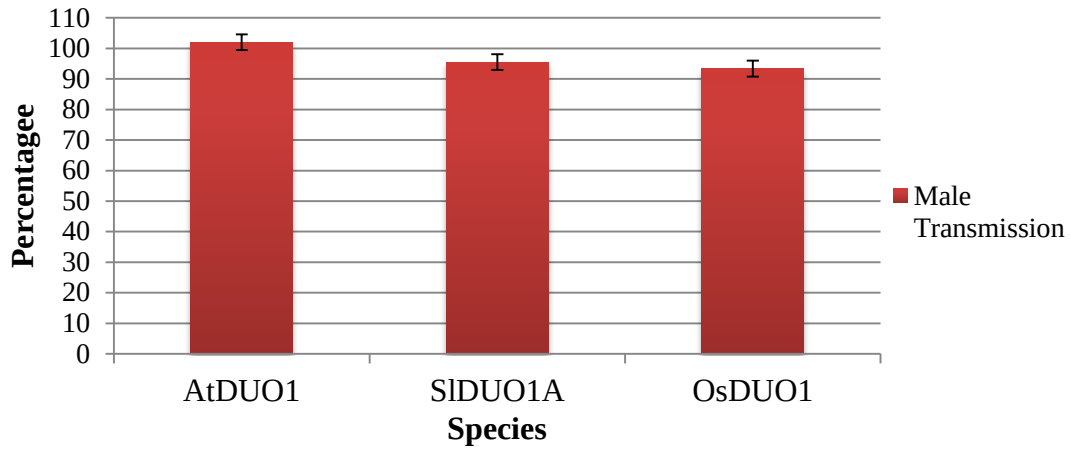


Figure 4.25: Transmission efficiency of the male transmission in species DUO1. A bar chart was used to illustrate the percentage of the transmission efficiency of the species. Control plant (AtDUO1) showed full transmission ratio. SlDUO1-A germ division ratio was lower than OsDUO1, however SlDUO1-A showed higher male transmission ratio than OsDUO1. All species DUO1 showed an ability to fertilize and transmit genetic information to the next generation. Error bars represent the standard error of the mean for the individuals analyzed.

4.9. Discussion

The findings from this study make several contributions to current knowledge. First of all the purpose of the project was to determine the conservation of DUO1 in various plants in different phylogenetic groups. *A.thailana* was the model plant as a eudicot, tomato as an other a divergent eudicot, rice as a monocot, and moss as an early land plant to understand of the DUO1 function in an evolutionary context. In order to show conservation of DUO1 in selected species, total RNA was extracted from various tissues including reproductive parts. According to RT-PCR analysis *DUO1* showed different expression levels in reproductive parts. After confirmation of expression of *DUO1* in reproductive tissues, another step, which was the cloning and sequencing of *DUO1* was carried out using two-step Gateway cloning. Sequencing results showed that *Arabidopsis*, tomato and rice DUO1 clones were perfectly matched with predicted sequences. Moss DUO1-A and B clone sequences showed different nucleotide sequences with predicted sequence. The *PpDUO1-A* sequence was 140bp shorter than predicted sequence. This study has demonstrated for the first time that moss *DUO1* gene predicted sequences were expressed in reproductive tissue. In all species R2R3 MYB domains were fully matched with predicted MYB domains that mean DUO1 was

conserved in all plants reproductive tissue. Taken together, these results suggest that DUO1 is male germline specific in angiosperms and reproductive part specific in land plants (since a mixture of tissues was used in the moss experiment described in this chapter).

A second contribution is transactivation by heterologous *DUO1* gene sequences using two target promoters, pDAZ1 and pMGH3. The greatest amount of activation by AtDUO1 was of pMGH3 compared with pDAZ1. SlDUO1-B construct displayed the lowest level of activation for both target promoters. The *OsDUO1* construct displayed a higher transactivation level than *SlDUO1-A* and *SlDUO1-B* of the pDAZ1. *OsDUO1* and *SlDUO1-A* constructs displayed similar levels of activation of pMGH3. However, the activity levels of two *PpDUO1-A* and *B* were not considerably different from the pMGH3. The results of this research support the idea that *Arabidopsis*, tomato and rice DUO1 are able to transactivate the *Arabidopsis* target promoters pMGH3 and pDAZ1

Recent study showed *PpDUO1-A* was activated in the presence of the Pp/At-Chimera DUO1. This suggests that the MYB domain of the *PpDUO1-A* may be able to bind the targets of its homolog in *A. thaliana*, but the activation of the targets will most likely require an effective C-terminus (Zhao, unpublished). This result also fits the previous experiments data on the *AtDUO1* C-terminus (Borg *et al.*, 2011). The results of this study support the idea that Arabidopsis, tomato and rice DUO1 can transactivate pMGH3 and pDAZ1.

Third, in order to assess the complementation potential of DUO1 orthologs from the selected species,*A. thaliana, S. lycopersicum, Oryza sativa*, and *Physcomitrella patens*, constructs were generated in which the *Arabidopsis* DUO1 promoter was used to drive expression of the CDS of the gene of interest fused to mCherry. Expression of *AtDUO1*, *OsDUO1* and *SlDUO1-A* transgenes increased the percentage of tricellular pollen to ~75 %, ~75%, and ~74% respectively. *SlDUO1-B* and *PpDUO1-A* did not show any expression of transgene and unable to increase the tricellular pollen (~50 %) in primary transformants (T1) plants. *PpDUO1-B* showed expression of transgene in some plants but was not able to rescue division failure of pollen. Thus expression of the *AtDUO1*, *SlDUO1-A*, and *OSDUO1* are able to fully complement, the mitotic *duo1* defect,

demonstrating conservation of function in *Arabidopsis*,tomato and rice. In contrast SlDUO1-B, PpDUO1-A and B fail to complement. This is the first time that the conservation of DUO1 function in germ cell division from other angiosperms has been explored and demonstrated.

The final part of the project was undertaken to try and establish if the *duo1* mutant pollen grains rescued with the DUO1 variants could also fertilize and transmit genetic information to the next generation. This was assayed by counting the frequency of *duo1* plants in the F1 generation resulting from cross between *ms1* pistils and duo1 pollen rescued by the selected species DUO1 variant transgenes. Control plant (*AtDUO1*) showed full transmission ratio. SlDUO1-A germ cell division ratio was lower than OsDUO1, however SlDUO1-A showed higher male transmission ratio than OsDUO1. The present study confirms previous finding which is *AtDUO1* is able to fully complement of the *duo1* phenotype in pollen (Borg *et al.*, 2011) and contributes additional evidence that suggests other angiosperm species DUO1 are able to fertilize and transmit genetic information to the next generation.

The empirical findings in this study provide a new understanding and evidence of the functional conservation of DUO1 from divergent species showing that the functions of DUO1 sequences are widely conserved among the angiosperms. Further research might explore 3' and 5' RACE (Rapid Amplification of cDNA Ends) approaches to get full-length sequences of *PpDUO1-A* and *PpDUO1-B* gene. Conservation of promoter identity could be explored by introducing the moss DUO1 promoters into *Arabidopsis*. Further research might explore in divergent species DUO1 orthologs genes in basal angiosperms and gymnosperms simply because now with the recent release of genome sequences for *Amborella* as a basal angiosperm (Amborella Genome Project, 2013).

Chapter 5

FUNCTIONAL CONSERVATION OF DAZ1 IN ANGIOSPERMS

5. Functional Conservation of *DAZ1* in Angiosperms

5.1. Summary

Background

Plants and animals possess a large number of C_2H_2 zinc finger transcription factors (TFs), many of which are considered to function as DNA binding proteins. C_2H_2 zinc finger domains are exhibited by around 0.7% of proteins in *Arabidopsis thaliana* (Englbrecht *et al.*, 2004) and during the development of male gametophytes, 60 proteins with C_2H_2 domains are expressed (Borges *et al.*, 2008; Honys & Twell, 2004). DUO1 directly activates a pair of redundant male germline-specific C_2H_2–type zinc finger proteins, DAZ1 and DAZ2, which play an important role in the division of germ cells and in gamete differentiation in *Arabidopsis* (Borg *et al.*, 2014). There is limited understanding of the regulation of male germline development at the molecular level in angiosperms, apart from in *A. thaliana*. Thus, it is great interest to investigate the functional conservation of DAZ1/DAZ2–related proteins in other angiosperms.

Results

Sequence conservation of *DAZ1/DAZ2* was examined in a wide range of angiosperms including *Arabidopsis thaliana*, *Solanum lycopersicum*, *Brassica rapa*, and the temperate grass model, *Brachypodium distachyon*. Expression of an AtDAZ1-mCherry fusion protein fully rescued the failed division phenotype of *Arabidopsis daz1/daz2* mutant pollen, whereas equivalent constructs with DAZ1 homologs from tomato (*SlDAZ1*) and *Brassica rapa* (*BrDAZ1b*) showed lower expression and limited, but significant rescue of failed division. *Brachypodium DAZ1 (BdDAZ1*) however, showed very weak transgene expression and did not rescue failed division. Transgenic lines expressing *DAZ1* orthologs were able to fully (*AtDAZ1*) or partially (SlDAZ1, BrDAZ1) rescue the transmission of *daz1/daz2* double mutant pollen, allowing *daz1/daz2* homozygous lines to be established harbouring heterologous *DAZ1* transgenes.

Conclusion

This study provides the first functional characterization of *DAZ1/DAZ2* orthologs in different angiosperms. *AtDAZ1* was able to fully and *SlDAZ1* and *BrDAZ1* to partially rescue the mitotic and transmission defects of *daz1/daz2* mutant pollen demonstrating conservation of function, whereas *BdDAZ1* did not show complementation, probably due to poor transgene expression.

5.2. Introduction

The twin sperm cells produced by the male gametophytes of flowering plants are vital for sexual reproduction through double fertilisation. The sperm cells develop in the male gametophyte from the progenitor generative cell, becoming specialised with regard to function and their ability to fertilise the female gametes. DUO1 is an essential transcription factor that is needed in the production of the functional twin sperm cells as it is required for the generative cell division and the differentiation of the sperm cells (Brownfield *et al.*, 2009a). The discovery of DUO1 also highlighted its role in the regulation of several important genes such as *GCS1* (Generative Cell Specific1) and *GEX2* (Gamete Expressed 2) which are relevant to the differentiation and function of sperm cells.

Despite this, the precise relationship that exists between the *CYCB1;1* (Cyclin-dependent protein kinase) and the DUO1 remains unknown. As has been reported in the past, dissimilar to the DUO1 target gene, *CYCB1;1* transcripts are not induced in seedlings as a result of ectopic DUO1 expression (Brownfield *et al.*, 2009a). The expression of *CYCB1;1* throughout the process of the cell cycle is subject to tight controls as it is only required during G2/M stage in order to stimulate mitotic division (Mironov *et al.*, 1999; Capron *et al.*, 2003). It remains a possibility that the DUO1 is not able to transcend these controls within seedlings, thus the authors suggests that the control of transcription of the *CYCB1;1* by DUO1 within the male germline may be controlled indirectly by post-transcriptional mechanisms (Brownfield *et al.*, 2009a).

The ability of DUO1 to ectopically trans-activate putative target genes employed available data for the Arabidopsis sperm cell transcriptome to screen for male germline expressed and specific transcripts (Borg *et al.*, 2011). A paralogous set of C_2H_2 zinc finger proteins, DUO1-Activated Zinc finger (DAZ), *DAZ1* (At2g17180) and DAZ2 (At4g35280) were among the 63 presumed DUO1 targets. RT-PCR on RNA extracted from dehisced pollen and sporophyte supports the transcriptome data pointing towards the pollen-specific expression of *DAZ1*/DAZ2 (Borg *et al.*, 2011; Pina *et al.*, 2005; Honys & Twell, 2004). Germline-specific activity was exhibited by *DAZ1*/*DAZ2* promoters when driving H2B::GFP, while *DAZ1*/DAZ2 mRNA levels were revealed by qRT-PCR to increase in bicellular pollen, peaking in tricellular pollen and declining in mature pollen (Borg *et al.*, 2011). The functions of *DAZ1* and *DAZ2* genes were characterised using T-DNA insertion lines *daz1-1* and *daz2-1* (Borg *et al.*, 2014). Single T-DNA knockouts in each gene revealed a wild-type phenotype. On the other hand, the heterozygous double knockout (*daz1-1 +/- daz2-2 -/-)* showed that generative cell was unable to divide, displaying similarities with the *duo1* phenotype in that the dehisced pollen was bicellular. Thus, it was concluded that *DAZ1* and *DAZ2* showed functional redundancy in germline development (Borg *et al.*, 2014). *DAZ1* and *DAZ2* are therefore a pair of zinc finger transcriptional repressors, which are activated by *DUO1* and show exclusive expression in the male germline (Borg *et al.*, 2014).

This chapter describes the functional analysis of *AtDAZ1* protein and its orthologues in *Solanum lycopersicum*, *Brassica rapa* and *Brachypodium distachyon* (monocot). The first section describes the validation of the predicted *DAZ1* sequences from genomic DNA of selected species by gateway cloning. Following this, the design of a transient luciferase assay was employed to quantify transactivation potential of *DAZ1* orthologues.The complementation potential of the *DAZ1* orthologs are then investigated using an *in vivo* pollen and the biological function of complemented *daz1/daz2* germ cells is examined by male transmission analyses. Finally, the identification and fertility of plants harbouring *DAZ1* orthologs in *daz1;daz2* double homozygous plants is described.

5.3. Identification of the *DAZ1* orthologs in selected angiosperms

The identification of homologs of *AtDAZ1* and *AtDAZ2* was performed using bioinformatics tools. BLAST (Basic Local Alignment Search Tool) of the Ensembl Plants database using the amino acids sequence for *AtDAZ1* and *AtDAZ2* gave a list of 37 proteins including *AtDAZ1* and *AtDAZ2*, all having an E-value ≤ 5.0x10-25. Included in the lists of homologs were a single gene from the *Solanum lycopersicum*, *Brachypodium distachyon*, and three genes from the *Brassica rapa.* Tomato *DAZ1* protein, which is Solyc06g060480.1 was named SlDAZ1. *Brachypodium* DAZ1 protein Bradi3g28850.1 was named BdDAZ1. Lastly, *Brassica* DAZ1 proteins, Bra011601, Bra017706, and Bra002027, were named as BrDAZ1a, BrDAZ1b, and BrDAZ1c respectively. Tomato, *Brachypodium*, and *Brassica rapa* DAZ1 consist of one exon and no introns similar to *AtDAZ1* and *AtDAZ2* (Figure 5.1). Thus, these species genomic DNA was used as a PCR template to generate products as follows, *SlDAZ1*, 810bp, *BdDAZ1*, 662bp, *BrDAZ1a*, *BrDAZ1b* and *BrDAZ1c*, 825bp, 879bp, and 525bp respectively. Tomato DAZ1 is 269 amino acids long and shows 40%, and 44% identity with *AtDAZ1* and *AtDAZ2* amino acid sequences. *Brachypodium distachyon DAZ1* is 222 amino acids long and shows 30% and 31% similarity with *AtDAZ1* and *AtDAZ2* . *Brassica rapa DAZ1* genes, *BrDAZ1a*, *BrDAZ1b* and *BrDAZ1c*, are 275, 292 and 174 amino acids long respectively. *BrDAZ1a*, and *BrDAZ1b* showed 58% similarity with *AtDAZ1* and 84% and 76% identity with *AtDAZ2* respectively.

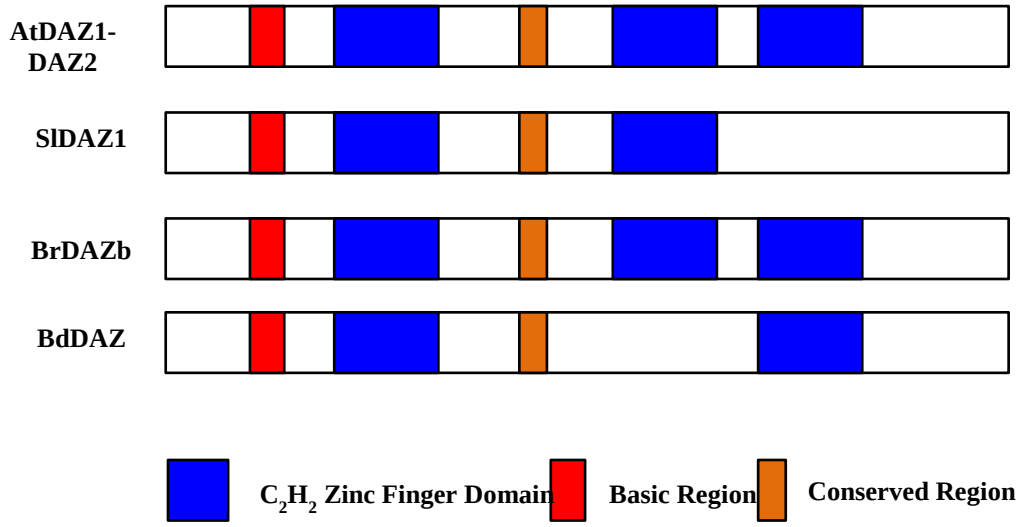


Figure 5.1: Schematic structure of the selected species DAZ. *AtDAZ1* and *AtDAZ2* have three C_2H_2 zinc finger domains and have the same basic arrangement. In *SlDAZ1* ZF3 (zinc finger 3) is missing and in *BdDAZ* ZF2 (zinc finger 2) is missing.

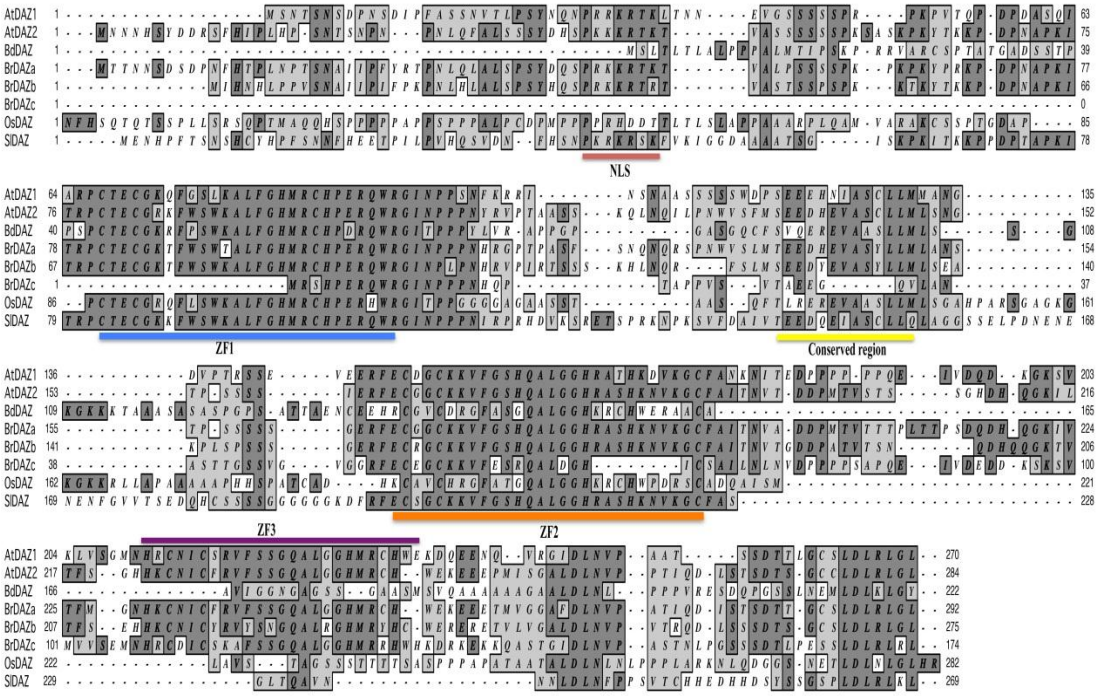


Figure 5.2: Alignment of selected angiosperm *DAZ1* and *DAZ2* homologs. Selected DAZ1/DAZ2 orthologs were aligned using ClustalW. *BrDAZ1c* has a substantial N-terminal deletion (up to the middle of the first zinc finger) and it likely to be non-functional. Conserved amino acids are bold in dark shaded boxes and similar amino acids are in light shaded boxes. Gene names 'a' and 'b' indicate paralogs. ZF1, ZF2, ZF3, zinc fingers 1-3, NLS, putative nuclear localization signal.

5.3.1. Cloning and sequencing of the selected DAZ1 orthologs

Genomic DNAs were extracted from *Solanum lycopersicum*'red setter', *Brachypodium distachyon*, and *Brassica rapa* (rapid cycling) leaves using a single leaf CTAB DNA extraction protocol. Primer pairs were designed and tested with BioTaq PCR to confirm gDNAs and primers gave the expected product sizes. For cloning, two step PCR was used with Velocity polymerase, since this enzyme has proof reading capacity. *SlDAZ*, *BdDAZ1*, and *BrDAZ*1 forward and reverse no stop and stop primer pairs were used with attB1 and attB2 sites to predict the expected product sizes in PCR-1 reaction. PCR results showed that primer pairs and gDNAs were reliable and thus, fragments were cloned by recombination based, Gateway cloning.

Once the expected band size was obtained it was excised from a gel and the second PCR was undertaken with attB1 and attB2 adapter primers, which correspond to the donor vector pDONR221. The predicted sized band was cut from the gel and extracted fragments for each were cloned into the Gateway vector pDONR221 and transformed into competent *E. coli* cells.

A colony screen was undertaken using generic M13 forward and reverse primers flanking the cloning site to confirm insertion of the correctly sized fragment in the vector. One colony was sent for sequencing from each species. Sequencing results for *SlDAZ1*, which was amplified using attB1SlDAZforward and attB2SlDAZreverse-no stop, showed one nucleotide change from that predicted, which did not however change the amino acid sequence, otherwise there was complete sequence alignment. *Brachypodium distachyon DAZ1* sequencing results showed perfect match with the predicted reference sequence in the ensemble database. *Brassica rapa* (rapid cycling) (*BrDAZ1b*) sequence results showed seven nucleotide changes compared with the reference sequences in the ensemble database (Table 5.1).

Table 5.1: Differences between the consensus and reference sequences in *B. rapa*. Seven nucleotide changes in cloned sequence gave rise to 3 amino acids changes and four silent mutations with predicted sequence of *B. rapa*. Four amino acid changes as follows; threonine (Thr) to lysine (Lys) at codon position 92; arginine (Arg) to glycine (Gly) at codon position 116; serine to cysteine at position161. Nt, Nucleotide.

Nt position	Nt change	Codon position	Codon change	Mutation/Amino acid change
225	C -> T	75	CCC -> CCT	Silent (Pro)
246	C -> T	82	ACC -> ACT	Silent (Thr)
275	C -> A	92	ACA -> AAA	Thr92Lys
346	C -> G	116	CGT -> GGT	Arg116Gly
480	T -> C	160	AGT -> AGC	Silent (Ser)
481	A -> T	161	AGT -> TGT	Ser161Cys
855	T -> A	285	TCT -> TCA	Silent (Ser)

5.4. *In planta* complementation analysis of DAZ1 variants

The identification of *DAZ* homologs has provided the opportunity to examine plant germline development and reproduction from an evolutionary perspective. The monocot-dicot disparity is a key juncture in the evolution of angiosperms. *Brassica rapa* and *A. thaliana* diverged 13-17 MYA (The *Brassica rapa* Genome Sequencing Project Consortium [BRGSPC], 2011). *Brachypodium*, a member of the Pooideae subfamily of Poaceae (grasses), is a wild annual grass endemic to the Mediterranean and Middle East that has promise as a model system. This has led to the development of highly efficient transformation, germplasm collections, genetic markers, a genetic linkage map and bacterial artificial chromosome (BAC) libraries. The genome sequence will allow *Brachypodium* to act as a powerful functional genomics resource for the grasses (The International *Brachypodium* Initiative, 2009). Despite the fact that the lineages of the *Arabidopsis* and the tomato families have approximately 112 million years separating them, how their corresponding genomes have diverged can be seen in the order and the number of the genes that they have in common. By comparing the genomes of *Arabidopsis* (Brassicaceae) and tomato (Solanaceae), a picture of how plant genomes have evolved can be drawn, since the radiation of eudicots plants, which is

relevant to important brassica and solanaceous crops. With *in vitro* protein expression and *in planta* complementation assays, it was discovered that the function of *DAZ1* has been maintained from a common ancestor of the monocot and dicot lineages.

5.4.1. Production of the DAZ1-mCherry fusion constructs for functional analysis from selected species

In order to evaluate the complementation potential of selected species *A. thaliana, S. lycopersicum, Brachypodium distachyon,* and *Brassica rapa*'rapid cyclin' DAZ constructs were generated in which the *Arabidopsis DAZ1* promoter was used to drive expression of an inserted gene CDS with mCherry fusion protein which are ProDAZ1:AtDAZ1-mCherry, ProDAZ1:SlDAZ-mCherry, ProDAZ1:BdDAZ-mCherry, ProDAZ1:BrDAZb-mCherry using 3-part gateway cloning system by Invitrogen. The reason to attach mCherry with DAZ constructs that transcription of *DAZ* could be visualized with Red fluorescence protein under UV light.

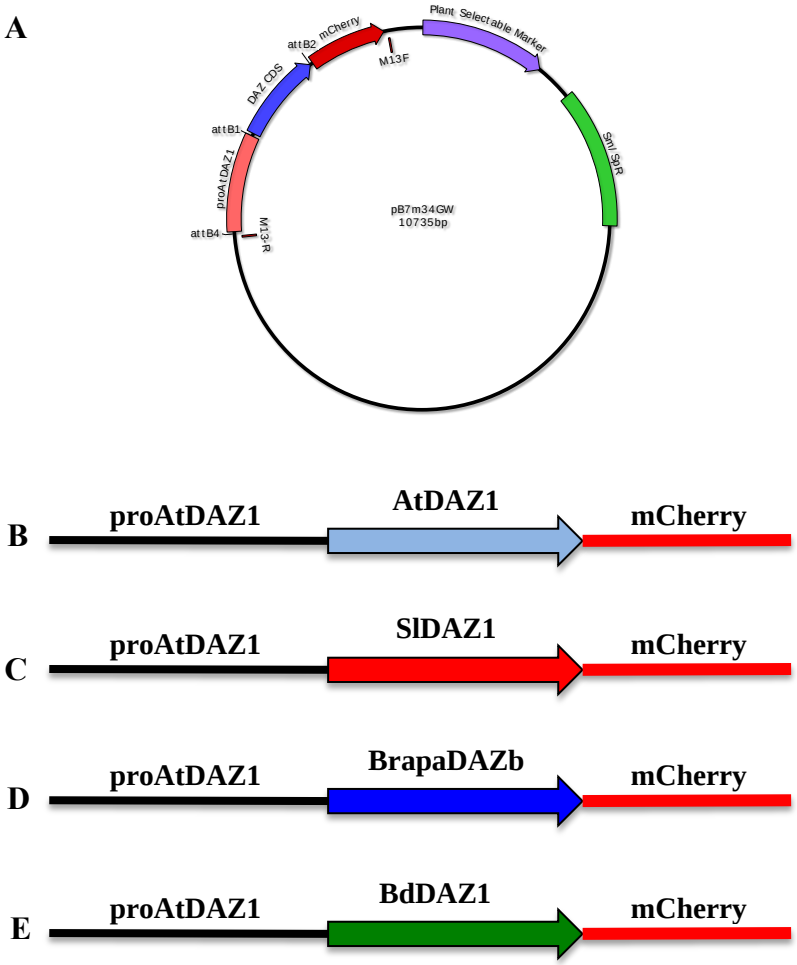


Figure 5.3: Schematic map of the species 3-part constructs. A: circular map image showed the structure of the 3-part construct with the destination vector pB7m34GW. Selected species constructs vectors of the four components were labeled B to E. These corresponded to AtDAZ1nostop-mCherry, SlDAZnostop-mCherry, BrDAZbnostop-mCherry, and BdDAZnostop-mCherry , and with promoter AtDAZ1 respectively. The 3-part constructs of the DAZ orthologs and homologues were mapped using MacVector software. “nostop” means this construct contains a translational fusion to the reporter gene.

5.4.2. *In planta* complementation analysis of DAZ1 homologs in selected species

To be able to analyse plants phenotypes, they supposed to be able accomplish transformation.Than, successfully transformed T1 plants seeds were sown and watered with Basta. Michael Borg generated ProDAZ1:AtDAZ1-mCherry construct and primary transformants (T1) screened for complementation of the *daz1* phenotype by Mihai Gherghinoiu. The plants *daz1/daz2* double hom het mutant (*daz1-1 -/- daz2-1 +/-*) have been transformed with pDAZ1-AtDAZ1-mCherry construct and T1 plants have been analysed. In the double mutant *daz1/daz2* hom-het the phenotype is 50% tricellular and 50% *bicellular* with the germ cell stuck in G2-M stage. We would predict that in the *daz1/daz2* double hom-het mutant transformed with pDAZ1-AtDAZ1-mCherry in T1 would have a 75% wild type phenotype and 25% daz1*/daz2* double hom-het phenotype. The additional 25% rescued pollen should harbour mCherry red fluorescent marker. The result of the complementation experiment reinforces the idea of the DAZ protein's role in the cell cycle or its role in rescuing mitotic division and promoting the cell in G2-M phase.

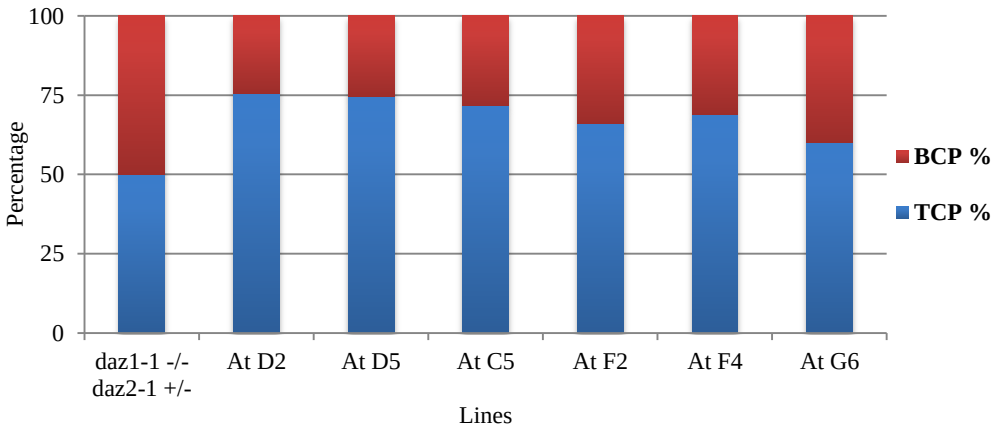


Figure.5.4: The protein fusion AtDAZ1-mCherry rescues *daz1/daz2* double het-hom mutant phenotype. A bar chart represented to describe the complementation potential of the AtDAZ1 construct to rescue the cell cycle defect in the *daz1* mutant. The examined Pollen from a daz1-1 +/- daz2-1 -/- plant without the transgene had ~50% tricellular pollen. Six individual plants which are single locus lines showing ~50% mCherry, have been analysed. D2, and D5 plants showed 75% tricellular pollen, C5, F2, F4 and G6 were showed 71.6%, 66%, 69%, and 60% respectively. Thus expression of the AtDAZ1 is able to complement the mitotic *daz1* defect and demonstrates conservation of function in *Arabidopsis*. BC: bicellular pollen, TC; tricellular pollen.

Other DAZ homologs expression constructs from selected species tomato, *Brachypodium*, and *Brassica rapa* were introduced *daz1-1 -/- daz2-1 +/-* plants. The T1 lines were primarily screened from lines to pinpoint representative *daz1* lines with a unique insertion for the transgene and a comparative mRFP signal which showed ~50% mCherry. With regards to the lines examined, the frequency of *daz1* pollen grains was established by scoring the number of bicellular and tricellular pollen grains by DAPI staining. Twenty-four specific plants of every construct were examined in isolation. From this first screening, specific lines were chosen for future research, especially those displaying higher than 50% daz1 and those appearing as tricellular pollen. *Brachypodium* lines generally showed weak transgene expression and number of pollen counts were not enough to validate its rescue. However, in order to confirm reproducibility of the transgene expression, T1 seeds was sown and analysed again, but at that time any mCherry transgene expression was observed. Tomato DAZ, and *Brassica rapa* DAZ showed partially rescued tricellular pollen.

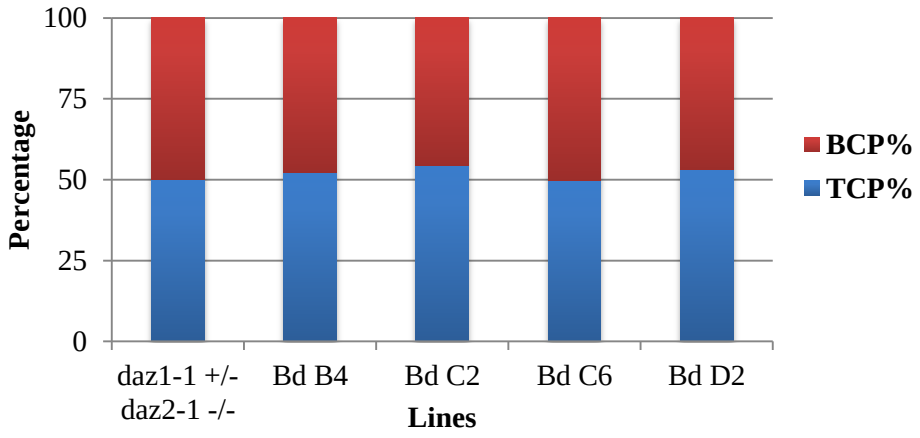


Figure 5.5: The protein fusion BdDAZ-mCherry partially rescues *daz1/daz2* double het-hom mutant phenotype. Four individual plants which are single locus lines showing ~50% mCherry, have been analysed. B4, C2, and D2 plants showed partially rescued tricellular pollen which were 52%, 54%, and 53% respectively. C6 total number of tricellular pollen population were showed ~50% . Thus expression of the BdDAZ is incompletely able to complement the mitotic *daz1* defect and demonstrates conservation of function in *Arabidopsis*. BCP: bicellular pollen, TCP; tricellular pollen.

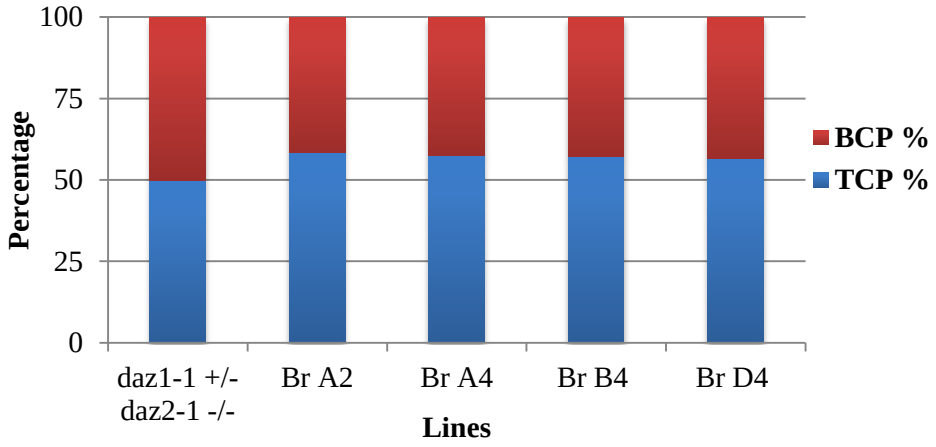


Figure 5.6: The protein fusion BrDAZb-mCherry partially rescues *daz1/daz2* double het-hom mutant phenotype. A bar chart represented to describe the complementation potential of the BrDAZb construct to rescue the cell cycle defect in the *daz1* mutant. Four individual plants which were single locus lines showing ~50% mCherry, have been analysed. A2, A4, B4, and D4 plants showed partially rescued tricellular pollen which were 58%, 57%, 57%, and 56% respectively. Thus expression of the BrDAZb is partially able to complement the mitotic *daz1* defect and demonstrates conservation of function in *Arabidopsis*. BCP: bicellular pollen, TCP; tricellular pollen.

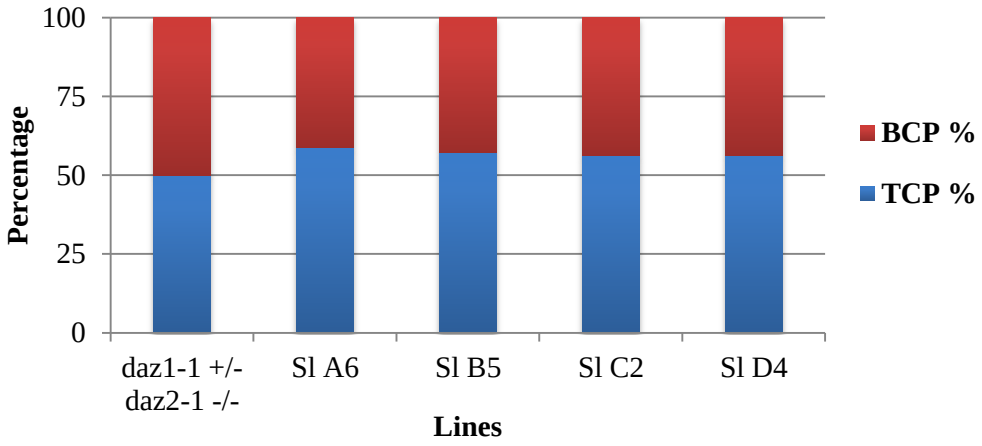


Figure 5.7: The protein fusion BrDAZb-mCherry partially rescues *daz1/daz2* double het-hom mutant phenotype. A bar chart represented to describe the complementation potential of the SlDAZ construct to rescue the cell cycle defect in the *daz1* mutant. Four individual plants which showed single locus lines showing ~50% mCherry, have been analysed. A6, B5, C2, and D4 plants showed partially rescued tricellular pollen, which were 58%, 57%, 56%, and 56% respectively. Thus expression of the SlDAZ is partially able to complement the mitotic *daz1* defect and demonstrates conservation of function in *Arabidopsis*. BCP: bicellular pollen, TCP; tricellular pollen.

Statistical analysis of the cell cycle rescue was undertaken using chi-square analyses, which evaluated differences in the examined ratio of wild type to *daz1* pollen grains from a 3:1 ratio (a fully-rescued single locus line) and a 1:1 ratio (a non-rescued line). Related chi-square evaluations were undertaken to evaluate the amount of *daz1* rescuing by testing deviations in the detected amount of tricellular pollen to bicellular pollen grains. In line with predictions, the pollen classes noticed in lines rescued with native DAZ protein displayed no deviation from a fully rescued line (3:1) but were quite different to a non-rescued line (1:1) for cell cycle rescue. As estimated, the pollen types observed in lines rescued with *AtDAZ1* protein were significantly difference from a fully rescued line (3:1) and non-rescued line (1:1), P-value (two-tailed) was calculated as 8.66143E-06, and 2.17959E-41respectively. SlDAZ, and BdDAZ, and BrDAZb protein showed highly significant differences from a fully rescued line (3:1) with 2.55431E-24, 3.07E-45, and 3.72212E-27 P- values respectively. Moreover, SlDAZ, and BrDAZb also showed significantly difference from a non-rescued line (1:1) with 0.000374844, and 8.86786E-05 P-values. BdDAZ protein showed no difference from a non-rescued line (1:1)(Table 5.2; Figure 5.8).

Table 5.2 Cell cycle rescue of complementing DAZ in selected species. Between four to six T1 lines expressing DAZ1-mCherry different transgenes in the *daz1-1 -/- daz2-1 +/-* plants total population pollen types were counted as a tricellular and bicellular pollen. Number of pollen types was shown in the table as a TCP and BCP. Chi-square test was evaluated to test for in the examined ratio of expected ratio of (3:1) if germ cell division was fully rescued. P-value <0.001, ***, ns; not significant, BCP; Bicellular pollen, TCP; Tricellular pollen.

						Based on a 3:1 ratio		Based on a 1:1 ratio	
Rescuing transgene	**Line**	**Total**	**TCP**	**BCP**	**TCP : BCP**	**χ^2 value**	**Significance**	**χ^2 value**	**Significance**
pDAZ1-AtDAZ1-Cherry	6	1201	834	367	2.3:1	19.8	***	181.6	***
pDAZ1-SlDAZ-Cherry	4	612	350	262	1.3:1	103.5	***	12.7	***
pDAZ1-BdDAZ-Cherry	4	731	383	348	1.1:1	199.2	***	1.7	ns
pDAZ1-BrDAZb-Cherry	4	704	404	300	1.3:1	116.5	***	15.4	***

5.5. Transmission efficiency of *daz1,daz2* pollen is rescued with *DAZ1* variants

The complementation of DAZ can act a source of extensive knowledge about DAZ function. This biological importance, as a result, is based on the production of twin sperm cells, which can successfully fertilize the female gametes and assist the creation of the next generation. Subsequently, a final experiment was undertaken to try and establish if the *daz1-1 -/- daz2-1 +/-* mutant pollen grains rescued with the DAZ variants could also fertilize and transmit genetic information to the resulting offspring. If the choice of lines satisfies the research, the transmission capacity of *daz* pollen taken with the DAZ variants was examined. Coming from the segregating population of every line, a *daz1-1 -/- daz2-1 +/-* mutant that was heterozygous for the transgene was crossed as pollen onto ms1 pistils. Under normal circumstances, the *daz1* mutation stops the pollen from going through fertilisation and as a result can only be maintained as a heterozygote. If a single copy transgene can completely rescue the *daz1* mutation, then half of the *duo1* pollen will include the transgene and thus have the ability to successfully fertilise and transmit. Because of this, *daz1* pollen without the transgene would not be able to transmit if the wild type pollen (25% with and 25% without the transgene) and the other 25% duo1 pollen containing the transgene were transmitting. As a result, the next generation would supposedly expect a 2:1 ratio of wild type plants (derived from the 50% wild type pollen) and *duo1* plants (derived from the 25% rescued *duo1* pollen).

F1 seeds were harvested from 4 lines for *Arabidopsis*, 2 lines for tomato, and 2 lines for *Brassica rapa*. Afterwards, F1 seeds were sown from *Arabidopsis* and tomato, and Brassica rapa on Phosphinothricin (PPT) (10 mg/ml) antibiotic selection plates with wild type negative control. Statistical analysis of the male transmission was undertaken with the help of chi-square analyses, which evaluated differences in the examined ratio of resistant to sensitive plants from a 2:1 ratio (a fully-transmitted line) and a 1:1 ratio (a non-transmitted line). Related chi-square evaluations were undertaken to evaluate the number of sensitive plants by testing deviations in the counted number of resistant to sensitive plants.

AtDAZ1 transmission efficiency showed no difference from a fully transmitted line (1.9:1) but was highly significantly different from a non-transmitted line (1:1). P-value (two-tailed) was calculated as 0.709548518 (2:1), and 4.39852E-13 (1:1). SlDAZ, and BrDAZb pollen transmission efficiency was significantly different from a fully transmitted line (2:1), P-value (two-tailed) was calculated as 0.00197483, and 0.001229385. However, they showed no difference from a non-transmitted line (1:1). Tomato and *Brassica rapa* DAZ showed similar transmission ratio (Table 5.3; Figure 5.8).

Table 5.3: Transmission efficiency of *DAZ1* homologues from selected species. Between two to four F1 line plants were counted as a resistant and sensitive. Numbers of resistant and sensitive plants were shown in the table as a PPT^R, and PPT^S. Chi-square test was evaluated to test for in the examined ratio of expected ratio of (2:1) if male transmission was fully transmitted. P-value <0.01, **, ns; not significant.

						Based on a 2:1 ratio		Based on a 1:1 ratio	
Rescuing transgene	**Line**	**Total**	**PPT^R**	**PPT^S**	**PPT^R:PPT^S**	**χ^2 value**	**Significance**	**χ^2 value**	**Significance**
pDAZ1-AtDAZ1-Cherry	4	519	342	177	1.9:1	0.139	ns	52.457	***
pDAZ1-SlDAZ-Cherry	2	158	87	71	1.2:1	9.573	**	1.620	ns
pDAZ1-BrDAZb-Cherry	2	184	102	82	1.2:1	10.446	**	2.174	ns

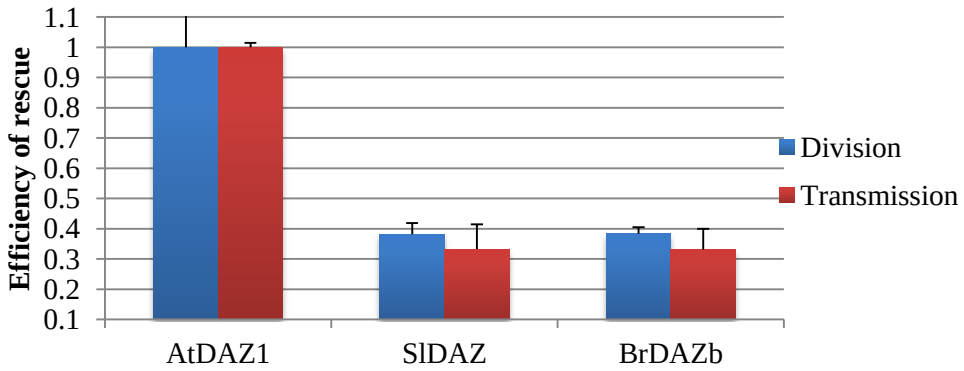


Figure 5.8: Functional analyses of the DAZ1 homologs by *in planta* complementation. The transgenes that were introduced in daz1-1 -/- daz2-1 +/- plants were pDAZ1-AtDAZ1-Cherry, pDAZ1-SlDAZ-Cherry, and pDAZ1-BrDAZb-Cherry. The proportion of tri-cellular pollen and the transmission of transgene-linked antibiotic resistance were established based on rescue of germ cell division and rescue of male transmission, respectively. Results are expressed as rescue efficiency against full-length AtDAZ1 protein, while error bars indicate the standard mean error. Division rescue and male transmission were analysed with n≥ 4 T1 lines and n ≥ 2 seedlings.

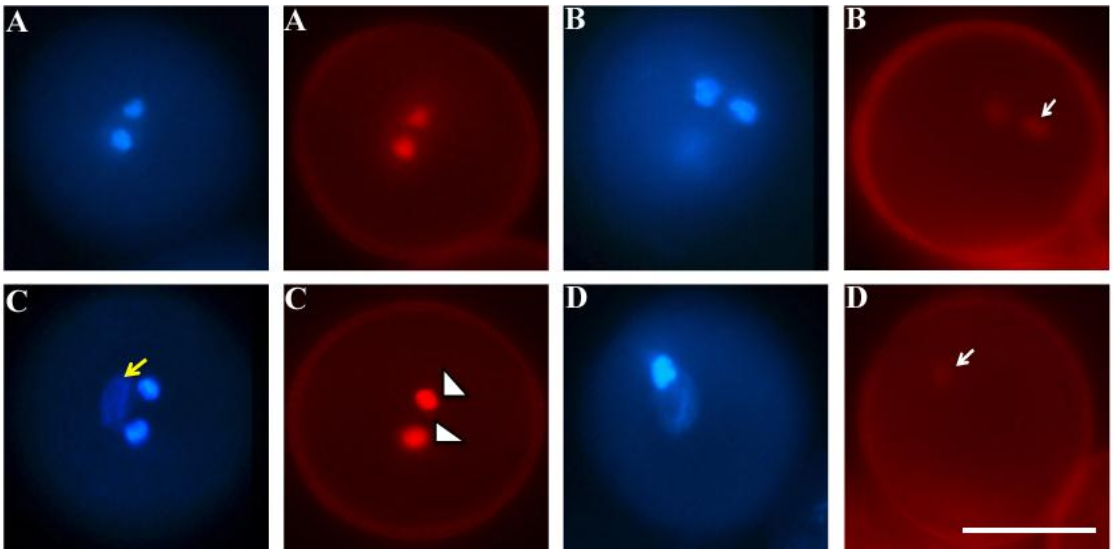


Figure 5.9: Images of the expression of T1 lines in selected species. Mature pollen from T1 lines harboring these constructs were stained with DAPI analyzed under RFP fluorescence. Images were shown for each construct from A to D that are ProDAZ1: AtDAZ1-mCherry, ProDAZ1:SlDAZ-mCherry, ProDAZ1:BrDAZb-mCherry,and ProDAZ1:BdDAZ-mCherry respectively. Tomato (B), and *Brachypodium* (D) DAZ construct showed very weak transgene expression that indicated by white arrows conversely *Arabidopsis* (A) and *Brassica rapa* (C) DAZ constructs showed high transgene expression. Yellow arrow shows vegetative cell, white triangles show sperm cells. Scale bar represents 10µm.

5.6. Characterisation of fertility in species DAZ

In order to identify the fertility of species *daz1 daz2* double mutant pollen, firstly, forty T2 plants phenotype have been analysed to find homozygous transgene (mCherry) for each species that are *A. thailana*, *S. lycopersicum*, and *B. rapa daz1-1-/- daz2-1+/-*. Then, selected lines gDNA was extracted from leaves using CTAB extraction method. After the extraction, multiplex PCR was performed with specific T-DNA insertion primer pairs for *DAZ1* and *DAZ2* to get double homozygous line for each species. The expected T-DNA insert was verified, and Michael Borg designed primers for each insert line.

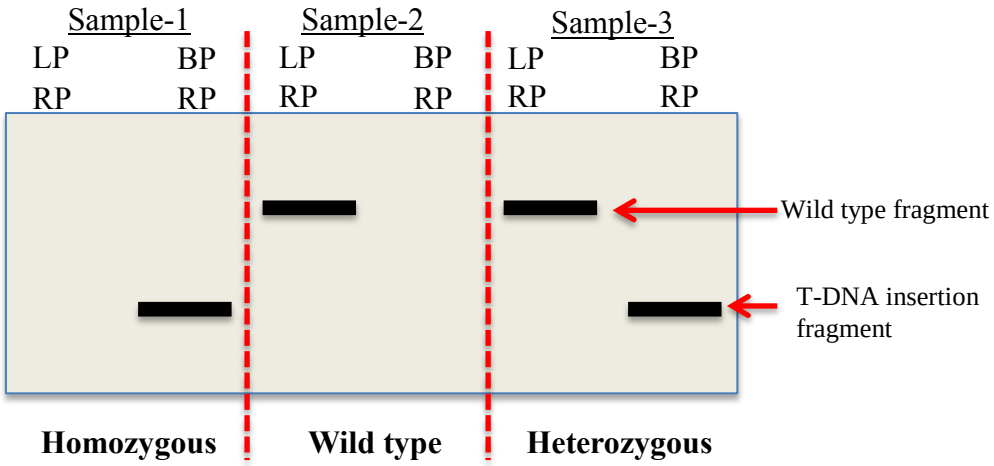


Figure 5.10: Illustration of predicted PCR products in genotyping T-DNA lines. If the plant is homozygous for the insert, only the PCR using the border primer (BP) and right primer (RP) produces a fragment. If the plant is wild type, only the PCR using the left primer (LP) and RP will produce a fragment. This is due to no insert being present for the BP primer to anneal to. If the plant is heterozygous, both primer sets will produce a fragment.

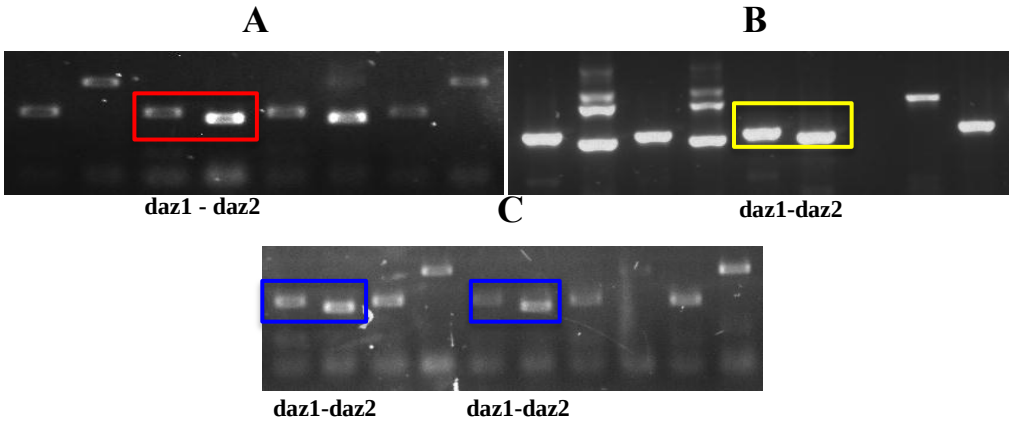


Figure 5.11: Images of the agarose gel of genotyped species. Multiplex PCR products were run on a 1.5% agarose gel to identify double homozygous *daz1-1 -/- daz2-1 -/-* in *Arabidopsis*, tomato and *Brassica rapa*. A, B, and C gel images are showing the genotyping of the *A. thailana, S. lycopersicum, and B. rapa* T2 generation respectively. One line *daz1-1 -/- daz2-1 -/-* (homozygous for *daz1* and homozygous for *daz2*) which are indicated in the red and yellow rectangles was found from *A. thailana, and S. lycopersicum* respectively. Two double homozygous lines that indicated in the blue rectangular were found from *B.rapa*.

A. thailana, *S. lycopersicum*, and *B. rapa* seeds were harvested and then one line seed was sown for each species as T3 generation to investigate fertility of the triple homozygous daz (*daz1-1 -/- daz2-1 -/-*,and homozygous transgene expression). Initially, 24 T3 generation plants were screened to confirm phenotypic character for each species, and as expected *AtDAZ1* triple homozygous lines have observed 100% tricellular pollen. SlDAZ, and BrDAZb triple homozygous lines showed approximately same percentage of tricellular pollen type with complementation rescue data. Columbia (WT) and *daz1 -/- daz2-1 +/-* lines without transgene were analysed as a control group (Figure 5.13).

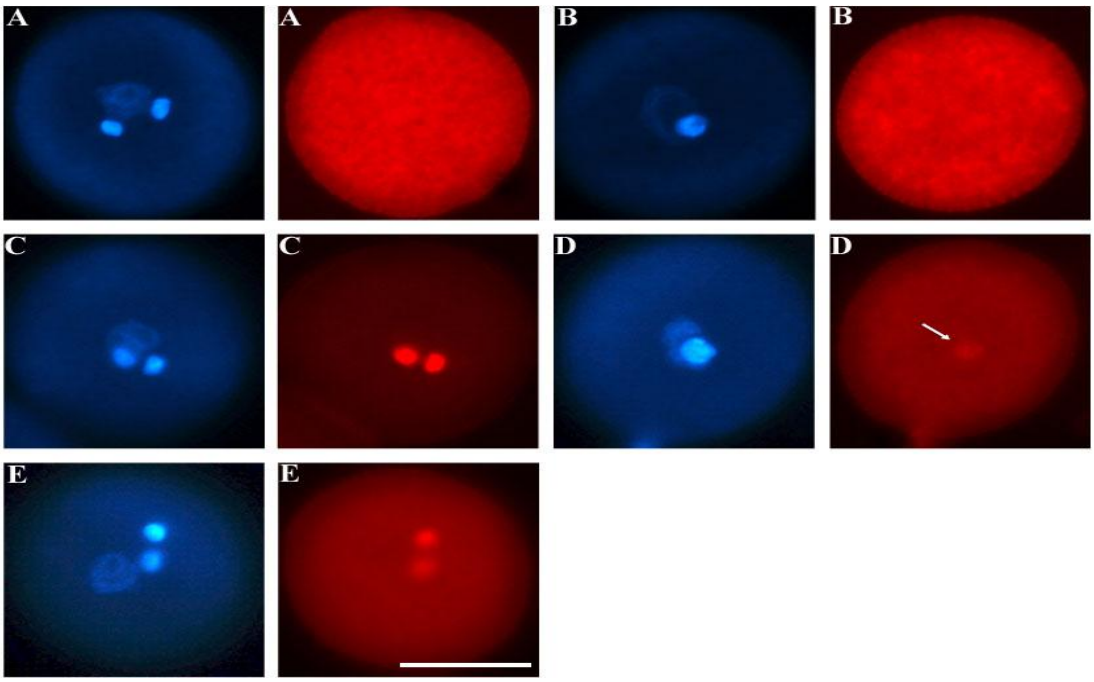


Figure 5.12: Images of the triple homozygous *daz1;daz2;DAZ1-mCherry* lines for selected species. Images show DAPI and RFP fluorescence for each construct from C to E, *A.thaliana*, *S. lycopersicum*, and *B. rapa* respectively. A, Col-0 (WT), B *daz1-1 -/- daz2-1 +/-* (untransformed control). Mature pollen from selected lines harboring these constructs was stained with DAPI and analyzed for RFP fluorescence. *SlDAZ-mCherry* (D) showed relatively weak transgene expression indicated with a white arrow. Scale bar represents 10μm

These triple homozygous transgenic lines showed significant differences in silique length and fertility. Fully complemented *AtDAZ1* siliques showed longer siliques than triple homozygous *SlDAZ1*, and *BrDAZ1b* siliques reflecting the incomplete complementation of *AtDAZ1*/*AtDAZ2* function by these orthologs genes (Figure 5.13;

Table 5.4).

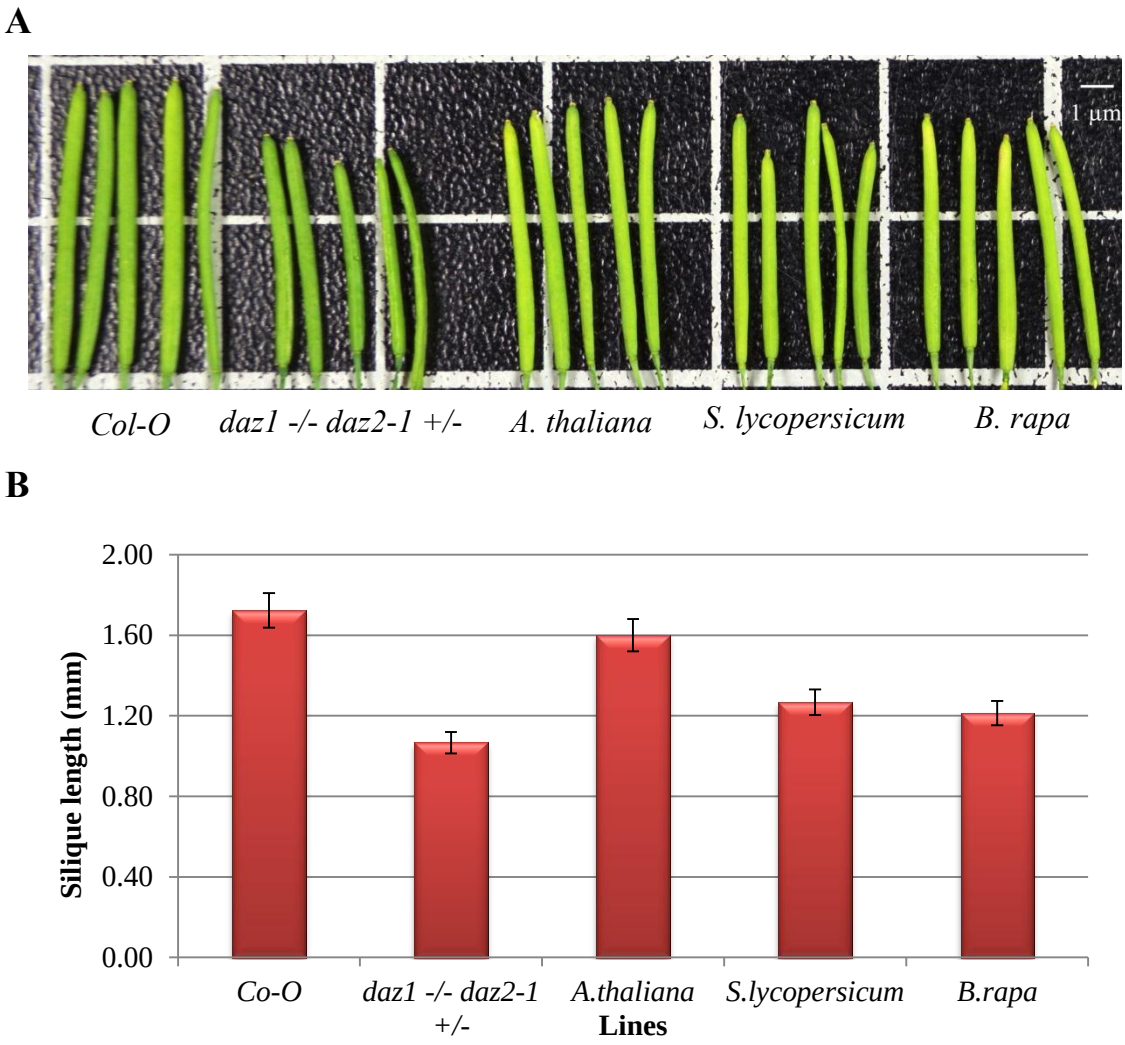


Figure 5.13: Morphological characters of the siliques in selected species. A: Image of representative siliques form 5 week old Col-0 (wildtype), *daz1-1 -/- daz2-1 +/-* , and triple homozygous transgenic lines rescued with *Arabidopsis*, tomato and *Brassica rapa* DAZ. B: bar chart showing mean silique length for three independent plants from transgenic and control plants. Ten siliques were measured for each plant. Col-0 siliques sizes ranged from 1.6 to 1.8 mm, *daz1-1 -/- daz2-1 +/-* , *A.thaliana*, *S.lycopersicum* ,and *B.rapa* siliques size ranged from 0.7 to 1.3mm, 1.5 to 1.8 mm, 1.1 to 1.6mm, and 1.1 to 1.3 mm were measured respectively. Error bars indicate the standard error . N=30 Scale bar represents 1µm.

In order to test statistical analysis of the siliques lengths between the species, two-paired t-test analyses was performed (Table 5.4).

Table 5.4: T-tests of silique lengths in heterologous *DAZ1* rescue lines. Col-O siliques length showed highly significance difference from a *daz1-1 -/- daz2-1 +/-* , *AtDAZ1*, *SlDAZ*, and *BrDAZb* triple homozygous lines. P-value was calculated as 1.54454E-18, 8.32624E-06, 7.88648E-14,and 6.59435E-20 respectively. According to ttest result, each species showed significantly difference each other in contrast to SlDAZ, and BrDAZb, which showed no significantly difference. P-value <0.001, ***, ns; not significant.

Paired T-test	P-value	Significance
Co-0-daz1 -/- daz2-1 +/-	1.54454E-18	***
Co-O-A.thaliana	8.32624E-06	***
Co-O-S.lycopersicum	7.88648E-14	***
Co-O-B.rapa	6.59435E-20	***
daz1 -/- daz2-1 +/- - A. thaliana	1.76946E-15	***
daz1 -/- daz2-1 +/- -S.lycopersicum	1.39871E-05	***
daz1 -/- daz2-1 +/- -B.rapa	4.50942E-05	***
A.thaliana- S.lycopersicum	5.20363E-11	***
A.thaliana-B.rapa	5.30346E-18	***
S.lycopersicum-B.rapa	0.050555103	ns

5.7. Discussion

This study provides the first functional characterization of *DAZ1*/*DAZ2* homologs in angiosperms. The aim of the project was to determine functional conservation of putative orthologs of *DAZ1*/*DAZ2* in various plants in different phylogenetic groups. *A. thailana* was the model plant as a eudicot, which tomato, and *Brassica rapa* were eudicot outgroups, and *Brachypodium distachyon* as a monocot (grass model). In order to show functional characterisation of *DAZ1* and *DAZ2* in selected species, total RNA was extracted from various tissues of *A. thaliana* and RT-PCR results showed that *DAZ1* and *DAZ2* were expressed only in pollen (Borg *et al.*, 2014). In addition, recent tissue specific RT-PCR showed *SlDAZ1* is expressed in pollen (Daud, unpublished). One of the main reasons to choose selected species DAZ gene is because DAZ is

intronless, so that gDNA can used as template for cloning. Sequencing results showed that, *S. lycopersicum* and *B. distahcyon DAZ* clones were perfectly matched to the predicted sequences. *Brassica rapa DAZb* clone sequences showed different nucleotide changes with predicted sequence. Seven nucleotide changes that gave rise to four amino acids change and three silent mutations were observed. This inconsistency may be due to using *Brassica rapa 'Rapid cycling'* gDNA as a template instead of *Brassica rapa* (Chinese cabbage). Taken together, these results suggest that DAZ is male germline specific for A. thailana (Borg *et al.*, 2014) and also DAZ conservation was validated in other angiosperms.

The second aim was to assess the complementation potential of DAZ homologs from selected species which *A. thaliana*, *S. lycopersicum*, *Brachypodium distachyon*, and *Brassica rapa'rapid cycling'* DAZ1. Constructs were generated in which the Arabidopsis DAZ1 promoter was used to drive expression of a CDS with mCherry fusion protein. In lines expressing the control construct ProDAZ1: AtDAZ1-mCherry, the number of tricellular pollen grains increased to ~75%. Conversely, expression of SlDAZ, BdDAZ, and BrDAZb transgenes increased the percentage of tricellular pollen to ~58% (ProDAZ1: SlDAZ-mCherry), 54% (ProDAZ1: BdDAZ-mCherry), and 56% (ProDAZ1: BrDAZb-mCherry), respectively. Phenotypes of transgenic plants were observed for various levels expression. *Arabidopsis* and *B. rapa* constructs showed high mCherry transgene expression in contrast to tomato, *B. distachyon*, and *Brassica rapa*'rapid cycling' constructs.

A possible explanation for this might be that the protein is not stable for selected angiosperms apart from *Arabidopsis* and *B.rapa*. In the case of *B. distachyon* complementation analysis, transgene expression was low to identify rescue pollen. It is difficult to explain this result, but it might be related to DAZ in the *B. distachyon* lack the second zinc finger domain found in the monocots. Also, among the eudicots tomato is unusual because it does not code for a third zinc finger domain. However, tomato *DAZ1* construct expressed well allowing analysis of several individuals to identify rescued pollen. Surprisingly, *B. rapa* was found to only partially complement the mitotic *daz1 daz2* defect. This result may be explained by the fact there are three *B.rapa DAZ* genes and all would need to be cloned and tested. Only BrDAZb was tested for complementation potential in this study. The overall results of this investigation show

that expression of *Arabidopsis* DAZ1 is able to fully complement, but tomato, *Brachypodium*, and *B. rapa* DAZ incomplete by complement the mitotic *daz1 daz2* defect so demonstrates conservation of function in flowering plants.

The third aim was undertaken to try and establish if the *daz1 daz2* mutant pollen grains rescued with the DAZ variants could also fertilize and transmit genetic information to the next generation. This was assayed by counting the frequency of herbicide resistant *daz1-1 -/- daz2-1 +/-* plants in the F1 generation resulting from cross between *ms1* pistils and *daz1 daz2* pollen rescued by the selected species *DAZ1* variant transgenes. Control plants (*AtDAZ1*) showed full transmission ratio (Borg *et al*., 2014). *SlDAZ* and *BrDAZb* germ cell division ratio were similar and also showed the same male transmission ratio that at 1.2:1. The present study confirms and contributes additional evidence that suggests selected angiosperms apart from *Arabidopsis* species *DAZ1* are able to partially fertilize and transmit genetic information to the next generation. The empirical findings in this study provide a new understanding and evidence of the functional conservation of DAZ from divergent species showing that the functions of DAZ sequences are widely conserved among the angiosperms.

A final experiment was undertaken to try to establish if the *daz1* mutant pollen grains rescued with the *DAZ1* orthologs could also fertilize and transmit the double *daz1/daz2* mutant alleles to the next generation through pollen. Plant double homozygous for *daz1* and *daz2* and homozygous for the 'species' DAZ1-mCherry fusion transgene were identified using genotyping. These triple homozygous transgenic lines showed significant differences in silique length and fertility. Fully complemented *AtDAZ1* siliques showed longer siliques than triple homozygous *SlDAZ1*, and *BrDAZ1b* siliques reflecting the incomplete complementation of *AtDAZ1*/*AtDAZ2* function by these orthologs genes.

Further research might explore in divergent species such as, *Amborella trichipoda*, *Physcomitrella patens* and rice to investigate functional conservation of DAZ in various plants. Another possible area of future research would be to investigate transactivation of *DAZ* genes in selected species which would provide valuable information about expression of *DAZ* in plants. The complementation potential of BdDAZ study is limited by the lack of information on screening transgene. Therefore, more research on the

ProDAZ1: BdDAZ-mCherry construct introduced in *daz* hom-het plant is required to determine the germ cell division and male transmission data. It is recommended that further research be undertaken to identify the fertility of species *daz1 daz2* double mutant pollen with homozygous transgene.

Chapter 6

GENERAL DISCUSSION

6. General Discussion

6.1. Biological significance of the coexistence polymorphic pollen

In terms of biological importance, both bi and tricellular pollen have many observable advantages. For example, whilst tricellular pollen may have a shorter lifespan, it is cellular primed for speed of germination (Brewbaker, 1967; Mulcahy & Mulcahy, 1988). Concerning bicellular pollen, studies on the pollen growth of certain species show that this growth is in fact taking place in two distinct phases, the first being relatively slow and autotrophic with no callose elements, and the subsequent phase being much faster and heterotrophic, with callose elements being produced in the pollen tubes (Mulcahy & Mulcahy, 1982). Tricellular pollen, on the other hand, appears to be dependent on external sources of nutrition from the very beginning of its growth (Mulcahy & Mulcahy, 1988). Its faster germination may prove valuable where this is of paramount importance. It is possible that the presence of three celled pollen is prevalent in certain types of dispersal mode, for example it has been observed that pollen spread by wind and water is more often tricellular and therefore more viable for quick germination (Knox, 1984). This faster germination could also prove invaluable as temperatures rise as this also accelerates the development of the female and thereby the entire reproductive cycle becomes faster (Sanzol & Herrero, 2001).

6.2. Evolutionary implication of the coexistence of polymorphic pollen in Magnoliaceae

Approximately 70% of angiosperms species contain bicellular pollen. An evolutionary derivative of bicellular pollen is tricellular pollen, which has been identified in a number of plant families (Brewbaker, 1967). By comparison to the observations made by Brewmaker during anther dehiscence, this study has found that in 27 out of 41 cases, Magnoliaceae species contained the polymorphic pollen type. It could thus be surmised that the bicellular pollen type is dominant in species belonging to the Magnoliaceae

family and in basal angiosperms (Brewbaker, 1967; Williams *et al.*, 2014). By contrast, it is only rarely that bicellular and tricellular pollen grains appear in the same genus (Brewbacker, 1967). Apart from Brewbaker, only a handful of other studies have reported the coexistence of the two pollen types in the same plant during anther dehiscence. Generally, such co-occurrence of pollen types has been observed in relatively old order species, including Laurales (Sampson, 1969; Gardner, 1974) and Alismatales (Grayum, 1985), in species of the families Olacaceae (Santalales) (Johri *et al*., 1992) and Euphorbiaceae (Malpighiales) (Webster & Rupert, 1973), as well as in contradictory studies of species of different genera like *Capsicum* (Lengel, 1960), *Populus* (Hamilton and Langridge, 1976), *Diospyros* (Sugiura *et al*., 1998), *Annonaceae* (Lora *et al.*, 2009), and *Saxifraga* (Zhang & Gornall, 2011). The present study detected polymorphic and bicellular pollen in *M. grandiflora* from different trees located in the same area. This suggests that, in addition to climate and location, pollen types in *M. grandiflora* may also be affected by genetics. Furthermore, pollen types also differ among the same species endogenous to different countries; for example, compared to the *Liriodendron tulipifera* collected from Leicester, where bicellular and tricellular pollen were observed, pollen collected from a South China Botanic Garden had solely bicellular pollen. Disparate results can also be obtained with different collections from the same place. For instance, over a period of three years, *Magnolia mulunica* was collected twice from the South China Botanic Garden. In the first collection, six hundred pollen grains contained tricellular pollen in a proportion of 0.3%. In the second collection in 2014, however, bicellular pollen was observed in the two hundred pollen grains from *Magnolia mulunica*. However, considering that the second collection was only a third of the first one, an increase in the pollen count might have led to the observation of tricellular pollen as well. Nevertheless, these findings indicate that collection time and location significantly influence the pollen type contained in species of Magnolia. The results of an experiment involving temperature regulation revealed that the amount of tricellular pollen at anthesis in *M. grandiflora* varies according to temperature. In a fluctuating environment, such temperature-regulated phenotypic plasticity in the proportion of tricellular to bicellular pollen could serve as a useful adaptation mechanism, modulating the timing of pollen access to the ovule in order to make sure that the egg cell is reached by some pollen tubes at the proper phase of development; this also lends credence to the notion that phenotypic plasticity has

contributed to evolution (Pigliucci, 2005). Therefore, tricellular pollen is likely to be produced in conditions where there are high levels of humidity in the proximity of the pollen and the temperature is conducive to generative cell division. This theory still requires close assessment, but Brewbaker (1967) proposed that aquatic taxa could benefit significantly from tri-cellular pollen. Furthermore, with the exception of Poaceae, tricellular pollen dispersal was observed in a more hydrated mode as well (Brewbaker, 1967; Knox, 1984).

6.3. Temperature effect is able to increase tricellular pollen in *M. grandiflora*

This research shows that bicellular and tricellular pollen coexist in anthers of *M.grandiflora* at dehiscence and their ratio in affected by various temperatures. Both types of pollen (bicellular and tricellular) can be considered as basic indicators of the occurrence of dehydration within the pollen itself, which may precede pollen mitosis II or occur subsequently. This might seem a reasonable explanation if the dehydration was not finalized when anther dehiscence is contiguous with high humidity. The division point between bicellular and tricellular pollen could primarily depend chronologically on the actions of pollen mitosis II when seen in the light of subsequent pollen shedding. The different timing of pollen mitosis (PMII) within the development of pollen has been noted (Friedman, 1999) and it is possible that my results could serve as a further proof of this fact. Experiments have highlighted the fact that the amounts of either bicellular or tricellular pollen within *M. grandiflora* can be affected and manipulated by altering the temperature level. This molecular flexibility in the bicellular to tricellular pollen ratio could provide the genus with a distinct advantage, namely the ability to respond to increasingly fluctuating environmental conditions.

6.4. *DUO1* is expressed and conserved in reproductive tissues.

The research project concentrated on the *DUO1* gene, which controls male gamete

production,and it was investigated the first example of male-germline specific gene (Rotman *et al.*, 2005). In conjunction with female gamete production, this forms the basis of sexual reproduction. The production of viable and fertile sperm is essential for reproduction and DUO1 manipulates the genes that make this feasible. Studying and comparing this gene within species three from distinct ancestral groups or clades has enabled an analysis of its origins, its functions and its inherent abilities over a chronological passage. This examination has however, been purely to study the gene's function, rather than its presence. This is primarily due to the fact that, whilst it is characteristic of all genes that they have ancestors and a chronological history, the existence of these is of little importence.

The importance of the *DUO1* gene in reproduction, its goals, methodology and the classification of its functions has led to a fascinating and credible reason for the examination that has been instigated as mentioned above (Durbarry *et al*., 2005, Rotman *et al*., 2005, Brownfield *et al*., 2009a, Brownfield, 2009b, Borg *et al*., 2009, Borg & Twell, 2010). The cloning of the gene *DUO1* from three species, *S. lycopersicum* (tomato), *O. sativa* (rice) and *P. patens* (moss), in addition to being an achievement in itself, also led to further queries being possible, which in turn leading to greater understanding. Comparisons of *A. thaliana*, *S. lycopersicum*, *O. sativa*, and *P. patens* have highlighted fundamental differences between the eudicots, monocots, and lower land plants and consequently the differences in adaptation and evolution that have taken place since they diverged (Rensink & Buell, 2004). This has thrown a scientific spotlight onto many points during the divergence at which major gene modification has taken place, and yet additional periods where entire gene clades have been conserved, both in their amino acid sequence and also in their consequent functions (Baumberger *et al.*, 2003). In this thesis one of the contributions is to confirm and show expression and conservation of *DUO1* in selected plants. Recent sequence analysis and transcriptomic data provide insight into our knowledge about gene expression. Selected species, tomato (Tomato Genome Consortium, 2012) as a divergent eudicot, rice (Project, International Rice Genome Sequencing, 2005) as a monocot, and moss (Rensing *et al.*, 2008) as an early land plant, all genomes have sequenced and this has enabled the extraction of *DUO1* sequences to validate its expression and conservation. This study has demonstrated, for the first time, that predicted tomato and moss *DUO1* homologs

sequences were validated in anther and antheridia-archegonia tissues. All species R2R3 MYB domains were fully matched with predicted MYB domains that suggests *DUO1* is conserved in all plants reproductive parts. This characteristic is unique to *DUO1* and its orthologs, as it is commonly conserved in divergent angiosperm groups from monocots to dicots. Therefore, it is logical to put forward the idea that its conservation is crucial from an evolutionary and functional perspective, but its importance has not been established empirically. Taken together, these results suggest that *DUO1* expression is specific to reproductive tissues in land plant and angiosperms.

6.5. Angiosperm DUO1 orthologs are functionally conserved.

Oryza sativa (rice) and *A. thaliana* can be employed to look at the difference of this split and explore 110-115 million years of evolution (Chaw *et al.*, 2004). Despite the fact that the lineages of the *Arabidopsis* and the tomato have approximately 112 million years ago between them, what their corresponding genomes have been undergoing since then can be seen in the order and the number of the genes that they have in common. The experiments detailed above showed DUO1 protein is conserved across dicots, monocots, and bryophytes. We were aware of many of the DUO1 targets such as MGH3, *DAZ1* etc. in *A. thaliana* (Borg, *et al.*, 2011). The subsequent activation of two of these by O. sativa and *S. lycopersicum* DUO1 orthologs *in vitro* tends to indicate strongly that it could be possible to manipulate the complicated transcriptional network of regulation observed in *A. thaliana*. PpDUO1A and PpDUO1-B were not able to activate pMGH3. However, a recent study showed PpDUO1-A was activated at the presence of the Pp/At-Chimera DUO1. This suggests that the MYB domain of the PpDUO1-A may be able to bind the targets of its homolog in *A. thaliana*, but the activation of the targets will most likely require an effective C-terminus (Zhao *et al.*, unpublished). This result also fits the previous experiments data on the AtDUO1 C-terminus (Borg *et al.*, 2011). This was then confirmed when its *in vivo* action was found to completely complement the SlDUO1-A, and OsDUO1 like AtDUO1 (Borg *et al.*, 2011). This leads us to believe that any modifications of DUO1 were formulated in advance of the divergence into monocot and dicot groups, a suggestion that is in fact

complemented by the studies of R2R3 MYBs in *Zea mays* (Maize) (Rabinowicz *et al.*, 1999). The ability to rescue germline division that has been identified for *A. thailana*, *S. lycopersicum*, and *O. sativa* DUO1 do not appear to be the same as those of the ancient moss *DUO1* homologs and SlDUO1-B. Some plants were observed mCherry transgene expression in tricellular and bicellular pollen population from PpDUO1-B line which means PpDUO1-B is being transcribed and protein is being produced. Despite the fact that the basic structure of DUO1 in *P. patens* will be totally kept for its own purposes, the conservation or substitution of amino acids within *A. thaliana, S. lycopersicum,* and *O. sativa* DUO1 will have redefined these for their own functional use in evolutionary terms, no matter the differences thus invoked. Although the MYB domain, which is highly conserved, is primarily responsible for DNA binding, it is the surviving C-terminal region that has the task of activating (Jin & Martin, 1999; Stracke *et al.*, 2001). Therefore it can be deduced, that as this is the area showing most differentiation between *P. patens* and other aligned species, an absence of the conservation of function is an appropriate conclusion.

The final experiment was designed to determine the efficacy of the DUO1 variants at rescuing the fertilization defect of *duo1* pollen grains. Howden *et al.*, (1998) highlighted that the transmission efficiency (TE) of mutant allele, or genes, via both the male and female gamete explains the small proportion of mutant genes that are effectively passed on to the next generation in contrast to the wild type allele. An evaluation of the transmission revealed that *duo1* most often transmitted via the female parent. It has been discovered that of 100% *Arabidopsis* and over 90% of tomato and rice *duo1* germ cells are capable of transmit via the female parents.

6.6. *Arabidopsis* DAZ1 is able to fully rescue the *daz1 daz2* mitotic defect while tomato and *B.rapa* DAZ1 partially rescue

DAZ1 and DAZ2 are members of the family of C_2H_2 zinc finger proteins (ZFPs), which comprises the largest number of transcription factors in *Arabidopsis thaliana*, according to the recent count based on *in silico* analysis (Englbrecht *et al.*, 2004). Three regulatory

proteins, namely DUO1, DAZ1 and DAZ2 as distinguished by research in *Arabidopsis* (Borg *et al*., 2011; Borg *et al*., 2014) perform essential roles in the development of both sperm cells. *DAZ1* and *DAZ2* mutants influence a number of aspects of the development and function of pollen. Mutants furthermore are male sterile, and distorted in segregation (Borg *et al*, 2014). Regardless, contemporary research has not currently covered any *DAZ* genes with comparable working in other angiosperms. *Arabidopsis* and other examples of angiosperms, which are distant evolutionary relatives, are *S. lycopersicum*, *Brassica rapa* and *B. distachyon*. Furthermore, *S. lycopersicum* and *Arabidopsis* produce mature pollen with differences. To identify tomato orthologs in DAZ1 and DAZ2 will enhance the understanding of maintenance mechanisms necessary in double fertilization. In spite of this, comprehending sperm cell development is essential for developments in plant breeding technologies such as hybrid seed production.One major example of this is the utilization of seedless products, producers now asked to focus on seedless fruits due to the partiality of consumers for seedless produce such as grapes, watermelon, citrus fruit and eggplant (Aleza *et al.*, 2009).

Considering both the bi-cellular phenotype of *DAZ1* and *DAZ2* mutated pollen and the reliance on *DUO1* for male germline expression powerfully supports the theory that DAZ1 and DAZ2 function downstream of DUO1 in the controlling of germ cell division. If this theory is correct the expression of *DAZ1* and *DAZ2* in *duo1* germ cells should rescue the *duo1* mitotic defect and produce pollen with twin sperm cells; and indeed this was shown to be the case (Borg *et al.*, 2014).

The ability of the complementation potential that have been identified of the *S. lycopersicum*, and *B. rapa* DAZ1 do not appear to be the same as those of the *A. thailana* (Borg *et al.*, 2014). Expression of an AtDAZ1-mCherry fusion protein fully rescued the failed division phenotype of *Arabidopsis daz1/daz2* mutant pollen, whereas equivalent constructs with *DAZ1* homologs from tomato (*SlDAZ1*) and *Brassica rapa* (*BrDAZ1b*) showed lower expression and limited, but significant rescue of failed division. It proves to be a challenge to understand the reasons behind the lack of division in *B. distachyon* DAZ1, however this may involve the fact that *B. distachyon* DAZ1 does not possess the second zinc finger domain that exists in the eudicots. Furthermore, eudicot tomato does not possess the third zinc finger domain. Such a

conclusion may be a result of the *B. rapa* DAZb gene failing to completely complement. Consequently, such a conclusion must be evaluated via the cloning of *B. rapa* DAZa and *B. rapa* DAZc genes. If the *daz1* mutant pollen grains rescued with the DAZ variants could also fertilise and transmit genetic information to the following generation. This understanding was assayed by counting the frequency of herbicide resistant *daz1-1 -/- daz2-1 +/-* plants in the F1 generation due to the cross of *msl* pistils and *daz1 daz2* pollen protected via chosen species of DAZ1 variant transgenes. Borg *et al.,* (2014) revealed a control specimen with complete ratio proportions. SIDAZ and BrDAZb germ cell division rates also correlated when highlighting equal male transmission proportions.

6.7. Conclusion

One major objective of the thesis was to assess the validity of Brewbaker's evidence that Magnoliaceae possess exclusively bicellular pollen at anthesis.The major finding is that 68% of Magnoliaceae species analysed showed polymorphic (bicellular and tricellular) pollen instead of exclusively bicellular pollen. Maturation of pollen under increased temperature regimes can significantly accelerate division of the generative cell in *M. grandiflora*.

A second major aim was to identify sequence and functional conservation of *DUO1* orthologs genes in angiosperms and bryophytes. DUO1 is conserved in selected species and shows preferential expression in reproductive organs. Expression of the rice DUO1 and tomato DUO1-A orthologs are able to complement the *duo1* generative cell division and transmission defect, which demonstrates a high level of conservation of DUO1 function in *Arabidopsis*. *Arabidopsis*, tomato and rice DUO1 orthologs were shown to transactivate *Arabidopsis* DUO1 target promoters. The expression of angiosperm DUO1 orthologs were able to complement the generative cell division and genetic transmission defects in *duo1* mutants, demonstrating conservation of function of DUO1 orthologs in *Arabidopsis.*

The final objective was to investigate the functional conservation of DAZ1/DAZ2–related proteins in other angiosperms. This study provides the first functional

characterization of *DAZ1/DAZ2* orthologs in different angiosperms. *AtDAZ1* was able to fully and *SlDAZ1* and *BrDAZ1* to partially rescue the mitotic and transmission defects of *daz1/daz2* mutant pollen demonstrating conservation of function, whereas *BdDAZ1* did not show complementation, probably due to poor the poor transgene expression.

7. Appendix

Table A1: Sequences of oligonucleotide primers used

Primer name	Primer 5'------->3' sequence
attB1F-adapter	GGGGACAAGTTTGTACAAAAAAGCAGGCT
attB2R-adapter	GGGGACCACTTTGTACAAGAAAGCTGGGT
DAZ1-TDNA-LP	TGATTTCGAAATGTGGAATGG
DAZ1-TDNA-RP	CAACAACTTCCACCCTGAATC
DAZ2-TDNA-LP	CAGATGCTTATGGCATTTTCTG
DAZ2-TDNA-RP	CTCATGTGACCAAAGAGAGCC
dCAPS SspI-fwd	GTACTCTGACTCACAGAATGATGAATAT
dCAPS-rvs	ACGGCTGAGGGAGAGCGAACAATGG
M13-F	GTAAAACGACGGCCAG
M13-R	CAGGAAACAGCTATGAC
Bra017706 attB1F	**ACAAAAAAGCAGGCTCT**ATGACCACCAATAATTCCG
Bra017706 attB2nsR	**ACAAGAAAGCTGGGTC**AAGCCCTAACCTAAGATCTAAAG
SlDAZattB1F	**ACAAAAAAGCAGGCTCT**ATGGAAAATCATCCTTTCAC
SlDAZattB2nsR	**ACAAGAAAGCTGGGTC**TAGCTTCAACCTCAAATCC
PpDUO1A-F	ATGAGCCTTAAGTCGGTGCAAAC
PpDUO1A-R	TGTATCGGCCGAAGCATCTGTG
PpDUO1B-F	GAGGGGCAGACAAAATGAGCGTA
PpDUO1B-R	TGACCCATGCCGCTACCAATTAC
attB1PpDUO1A-F	**ACAAAAAAGCAGGCTCT**ATGAGCCTTAAGTCGGTGCAAAC
attB2PpDUO1A-Rnstop	**ACAAGAAAGCTGGGTC**TTGCGCAACCCACCACTCG
attB2PpDUO1A-Rstop	**ACAAGAAAGCTGGGTC**TCATTGCGCAACCCACCACT
attB1PpDUO1B- F	**ACAAAAAAGCAGGCTCT**ATGAGATCGACAAAATTGAGTTCA
attB2PpDUO1BRstop	**ACAAGAAAGCTGGGTC**AATGGTGGGTTGCGCAATAA
attB2PpDUO1BRnstop	**ACAAGAAAGCTGGGTC**AGAATGGTGGGTTGCGCAA
SlDUO1 chr1- F	AAGTCTTGTCGTCTTCGTTG
SlDUO1 chr1 -R	GGCACTGAGTTTCGTAGAATC
SlDUO1 chr10-F	GGAGAAGAAGAAAGGACAGTG
SlDUO1 chr10-R	CCTGATGTGAAGTTGATGATGC
SlActin-F	TTGCTGACCGTATGAGCAAG
SlActin-R	GGACAATGGATGGACCAGAC

Table A2: frequency of bicellular and tricellular pollen in Magnoliaceae species from China.II=Bicellular, III= Tricellular, PM= Polymorphic, SCBG=South China Botanical Garden, KMBG=KunMing Botanical Garden

Species Name	BCP(n)	TCP(n)	% BCP	%TCP	Pollen type	Source
Magnolia mulunica	628	2	99.68	0.3	PM	SCBG
Magnolia paenata	585	0	100	0.0	II	SCBG
Magnolia championii	435	25	94.57	5.4	PM	SCBG
Magnolia albosericea	480	20	95.84	4.2	PM	SCBG
Magnolia henryi	335	14	95.83	4.2	PM	SCBG
Magnolia shangsiensis	370	4	98.92	1.1	PM	SCBG
Magnolia tripetala	154	19	89.02	11.0	PM	KMBG
Michelia sirindhoniae	250	4	98.43	1.6	PM	SCBG
Michelia crassipes	410	0	100	0.0	II	SCBG
Manglietia wongtungensis	529	16	97.07	2.9	PM	SCBG

Table A3: Frequency of bicellular and tricellular pollen in Magnoliaceae species from various sources. II=Bicellular, III= Tricellular, PM= Polymorphic.

Species Name	BCP(n)	TCP(n)	%TCP	Pollen type	Source
Magnolia floribunda	120	0	0	II	SFBG
Magnolia virgiana var australis	120	3	2.4	PM	SFBG
Magnolia denudata	150	0	0.0	II	NYBG
Magnolia bailonii	150	0	0.0	II	China
Magnolia manudiae	200	0	0.0	II	China
Magnolia cavaleriae	150	0	0.0	II	SFBG
Magnolia laevitolia	125	6	4.6	PM	SFBG
Magnolia doltsopa	100	3	2.9	PM	SFBG
Magnolia liliiflora	112	9	7.4	PM	SFBG
Michelia yunnansis	150	0	0.0	II	China
Magnolia mulinica	150	0	0.0	II	China
Magnolia paeneatalauma	150	0	0.0	II	China
Michelia alba	100	7	6.5	PM	China
Magnolia kobus	120	7	5.5	PM	NYBG
Magnolia virginiana	150	0	0.0	II	NYBG
Liriodendron chinensis	150	0	0.0	II	China
Liriodendron tulipifera	150	0	0.0	II	China
Liriodendron tulipifera	160	40	20.0	PM	Leicester

Table A4: Frequency of bicellular and tricellular pollen in Magnoliaceae species from Leicester. II=Bicellular, PM= Polymorphic, UOLBG; University of Leicester botanic garden.

Species Name	BCP(n)	TCP(n)	%TCP	Pollen type	Source
Magnolia salicifolia	390	8	2.0	PM	UOLBG
Magnolia stellata	208	12	5.5	PM	UOLBG
Magnolia seiboldii	641	7	1.1	PM	UOLBG
Magnolia wilsonii	341	7	2.0	PM	UOLBG
Magnolia campbellii	655	0	0.0	II	UOLBG
Magnolia cylindrica	225	22	8.9	PM	UOLBG

Table A5: Frequency of tricellular pollen of the temperature treatment of *M.grandiflora* flower-1 and flower-2. During fives days, stamens were taken and fixed with fixative. Than, pollen phenotype was counted. Three days later, stamens, which were in the 30^{o}C growth chamber were dehiscent and also showed the highest percentage of tricellular pollen (19.0-19.3 %) in that temperature regime. A; flower-1, B; flower-2

A

1.Count

	1.day			
	Total(n)	BCP	TCP	%TCP
15C	130	130	0	0.00
20C	186	185	1	0.54
25C	157	155	2	1.27
30C	114	112	2	1.75

	2.day			
	Total(n)	BCP	TCP	%TCP
15C	179	168	11	6.15
20C	154	142	12	7.79
25C	160	140	20	12.5
30C	162	139	23	14.2

	3.day			
	Total(n)	BCP	TCP	%TCP
15C	164	152	12	7.32
20C	165	149	16	9.70
25C	165	144	21	12.73
30C	163	132	31	19.02 *

	4.day			
	Total(n)	BCP	TCP	%TCP
15C	150	132	18	12
20C	155	130	25	16.13 *
25C	173	141	32	18.50 *
30C	135	109	26	19.26

	5.day			
	Total(n)	BCP	TCP	%TCP
15C	161	136	25	15.53 *
20C	210	175	35	16.67
25C	192	157	35	18.23
30C	166	134	32	19.28

2.Count

	1.day			
	Total(n)	BCP	TCP	%TCP
15C	180	180	0	0.00
20C	200	200	0	0
25C	140	138	2	1.43
30C	143	139	4	2.80

	2.day			
	Total(n)	BCP	TCP	%TCP
15C	172	162	10	5.81
20C	151	140	11	7.28
25C	146	130	16	10.96
30C	133	114	19	14.29

	3.day			
	Total(n)	BCP	TCP	%TCP
15C	150	140	10	6.67
20C	165	150	15	9.09
25C	174	152	22	12.64
30C	157	127	30	19.11

	4.day			
	Total(n)	BCP	TCP	%TCP
15C	130	116	14	10.77
20C	152	126	26	17.11
25C	159	130	29	18.24
30C	150	120	30	20

	5.day			
	Total(n)	BCP	TCP	%TCP
15C	146	128	18	12.33
20C	175	145	30	17.14
25C	159	130	29	18.24
30C	152	122	30	19.74

3.Count

	1.day			
	Total(n)	BCP	TCP	%TCP
15C	200	200	0	0.00
20C	171	170	1	0.58
25C	117	116	1	0.85
30C	130	127	3	2.31

	2.day			
	Total(n)	BCP	TCP	%TCP
15C	152	143	9	5.9
20C	126	116	10	7.94
25C	132	115	17	12.88
30C	111	96	15	13.51

	3.day			
	Total(n)	BCP	TCP	%TCP
15C	94	87	7	7.45
20C	137	123	14	10.22
25C	166	146	20	12.05
30C	156	125	31	19.87

	4.day			
	Total(n)	BCP	TCP	%TCP
15C	158	141	17	10.76
20C	155	129	26	16.77
25C	166	135	31	18.67
30C	142	114	28	19.72

	5.day			
	Total(n)	BCP	TCP	%TCP
15C	158	136	22	13.92
20C	152	127	25	16.45
25C	162	132	30	18.52
30C	170	136	34	20

Mean

	1.day			
	Total(n)	BCP	TCP	%TCP
15C	170	170	0	0
20C	185.7	185	0.67	0.37
25C	138	136.3	1.67	1.19
30C	129	126	3	2.29

	2.day			
	Total(n)	BCP	TCP	%TCP
15C	167.7	157.7	10	5.96
20C	143.7	132.7	11	7.67
25C	146	128.3	17.7	12.11
30C	135.3	116.3	19	14.00

	3.day			
	Total(n)	BCP	TCP	%TCP
15C	136	126.3	9.7	7.14
20C	155.7	140.7	15	9.67
25C	168.3	147.3	21	12.47
30C	158.7	128	30.7	19.33

	4.day			
	Total(n)	BCP	TCP	%TCP
15C	146	129.7	16.3	11.18
20C	154	128.3	25.7	16.67
25C	166	135.3	30.7	18.47
30C	142.3	114.3	28.0	19.66

	5.day			
	Total(n)	BCP	TCP	%TCP
15C	155	133.3	21.7	13.93
20C	179	149	30	16.75
25C	171	139.7	31.3	18.33
30C	162.7	130.7	32	19.67

B

1.count

	1.day			
	Total(n)	BCP	TCP	%TCP
15C	144	134	10	6.9
20C	151	140	11	7.3
25C	149	132	17	11.4
30C	150	133	17	11.3

	2.day			
	Total(n)	BCP	TCP	%TCP
15C	177	161	16	9.0
20C	145	125	20	13.8
25C	139	115	24	17.3
30C	147	121	26	17.7

	3.day				
	Total(n)	BCP	TCP	%TCP	
15C	186	161	25	13.4	
20C	107	92	15	14.0	
25C	135	110	25	18.5	*
30C	131	105	26	19.8	*

	4.day			
	Total(n)	BCP	TCP	%TCP
15C	180	150	25	13.89
20C	153	130	23	15.03
25C	167	136	31	18.56
30C	210	168	42	20.00

	5.day				
	Total(n)	BCP	TCP	%TCP	
15C	127	107	20	15.75	*
20C	162	135	27	16.67	*
25C	154	125	29	18.83	
30C	205	164	41	20	

2.Count

	1.day			
	Total(n)	BCP	TCP	%TCP
15C	143	135	8	5.59
20C	149	138	11	7.38
25C	151	135	16	10.60
30C	145	128	17	11.72

	2.day			
	Total(n)	BCP	TCP	%TCP
15C	160	147	13	8.13
20C	152	132	20	13.16
25C	154	128	26	16.88
30C	146	120	26	17.81

	3.day			
	Total(n)	BCP	TCP	%TCP
15C	169	150	19	11.24
20C	123	105	18	14.63
25C	138	113	25	18.12
30C	156	125	31	19.87

	4.day			
	Total(n)	BCP	TCP	%TCP
15C	134	115	19	14.18
20C	149	126	23	15.44
25C	172	141	31	18.02
30C	162	128	34	20.99

	5.day			
	Total(n)	BCP	TCP	%TCP
15C	140	120	20	14.29
20C	155	130	25	16.13
25C	143	117	26	18.18
30C	150	120	30	20.00

3.Count

	1.day			
	Total(n)	BCP	TCP	%TCP
15C	164	153	11	6.71
20C	150	127	13	8.67
25C	147	131	16	10.88
30C	150	133	17	11.33

	2.day			
	Total(n)	BCP	TCP	%TCP
15C	171	156	15	8.8
20C	156	136	20	12.8
25C	153	128	25	16.3
30C	155	127	28	18.1

	3.day			
	Total(n)	BCP	TCP	%TCP
15C	162	142	20	12.35
20C	135	116	19	14.07
25C	143	117	26	18.18
30C	138	110	28	20.29

	4.day			
	Total(n)	BCP	TCP	%TCP
15C	146	126	20	13.70
20C	155	130	25	16.13
25C	151	123	28	18.54
30C	168	134	34	20.24

	5.day			
	Total(n)	BCP	TCP	%TCP
15C	129	110	19	14.73
20C	163	136	27	16.56
25C	148	120	28	18.92
30C	148	118	30	20.27

Mean

	1.day			
	Total(n)	BCP	TCP	%TCP
15C	150.3	140.7	9.7	6.42
20C	150	135	11.7	7.78
25C	149	132.7	16.3	10.96
30C	148.3	131.3	17	11.46

	2.day			
	Total(n)	BCP	TCP	%TCP
15C	169.3	154.7	14.7	8.65
20C	151	131	20	13.26
25C	148.7	123.7	25	16.83
30C	149.3	122.7	26.7	17.85

	3.day			
	Total(n)	BCP	TCP	%TCP
15C	172.3	151	21.3	12.34
20C	121.7	104.3	17.3	14.24
25C	138.7	113.3	25.3	18.27
30C	141.7	113.3	28.3	20.00

	4.day			
	Total(n)	BCP	TCP	%TCP
15C	153.3	130.3	21.3	13.92
20C	152.3	128.7	23.7	15.53
25C	163.3	133.3	30	18.38
30C	180	143.3	36.7	20.41

	5.day			
	Total(n)	BCP	TCP	%TCP
15C	132	112.3	19.7	14.92
20C	160	133.7	26.3	16.45
25C	148.3	120.7	27.7	18.64
30C	167.7	134	33.7	20.09

Table A6: Frequency of the rescue of germ cell division in selected species DUO1

AtDUO1		**RFP+ (n)**	**%RFP (%)**	**RFP- (n)**	**RFP (%)**	**Total (n)**	**TCP (n)**	**TCP (%)**	**BCP (n)**	**% BCP**
B4 Line - B1	50	50	50	50	100	116	75.3	38.0	24.7	**Total (n)**
B4 Line - B5	50	50	50	50	100	89	76.1	28.0	23.9	154
B4 Line - C1	50	50	50	50	100	119	74.8	40.0	25.2	117
B4 Line- C6	50	50	50	50	100	114	75.5	37.0	24.5	159

SlDUO1-Chr1	**TCP RFP+**	**TCP, RFP-**	**BCP, RFP+**	**BCP, RFP-**	**Total (n)**	**TCP RFP+ %**	**TCP, RFP-%**	**BCP, RFP+%**	**BCP, RFP-**	581	
C1	42	40	**10**	22	114	36.84	35.09	8.77	19.30	**TCP %**	**BC P%**
D3	68	44	11	27	150	45.33	29.33	7.33	18.00	72	28
D5	65	37	11	26	139	46.76	26.62	7.91	18.71	75	25
										73	27

OsDUO1	**RFP+ (n)**	**%RFP (%)**	**RFP- (n)**	**RFP (%)**	**Total (n)**	**TCP (n)**	**TCP (%)**	**BCP (n)**	**% BCP**	
C3 Line - A3	50	50.0	50	50.0	100	114	75	38	25.0	**Total (n)**
C3 Line - A5	50	50.0	50	50.0	100	101	74.8	34	25.2	152
B6 Line - A2	50	50	50	50	100	81	75.0	27	25.0	135
B6 Lin - A3	50	50	50	50	100	93	75.0	31	25.0	108

Table A7: Frequency of the rescue of male transmission in selected species DUO1

AtDUO1 F1 seed							
Line	pptR	pptS	ungerminated	total	Sum	% R	%S
B4/B5	34	16		50	50	68	32
B4/B5	32	17	1	50	49	65.31	34.69
b4/B1	33	16	1	50	49	67.35	32.65
b4/B1	33	16	1	50	49	67.35	32.65
	132	65					
SlDUO1-A F1	pptR	pptS	ug	total	Sum	% R	%S
C1 line	60	33	7	100	93	64.52	35.48
C1 line	30	20	0	50	50	60.00	40.00
C1 line	38	19	3	60	57	66.67	33.33
D3 line	36	17		53	53	67.92	32.08
D3 line	37	20		57	57	64.91	35.09
D3 line	32	16	2	50	48	66.67	33.33
D5 line	36	17	2	55	53	67.92	32.08
D5 line	32	16	2	50	48	66.67	33.33
D5 line	34	16	0	50	50	68	32
	335	174		525			
OsDUO1 F1 seed							
Line	pptR	pptS	ug	total	Sum	% R	%S
c3/A3	30	16	4	50	46	65.22	34.78
c3/A3	25	13	12	50	38	65.79	34.21
b6/A2	31	17	2	50	48	64.58	35.42
b6/A2	32	16	2	50	48	66.67	33.33
	118	62					

Table A8: Frequency of the rescue of germ cell division in selected species DAZ1

Division rescue analysis

Sample	WT +	WT -	*daz*+	*daz* -	Total	TCP	BCP	%TCP	%BCP	RFP +	RFP -	% RFP +	% RFP -
At D2	90	61	14	35	200	151	49	75.5	24.5	104	96	52	48
At D5	106	43	3	48	200	149	51	74.5	25.5	109	91	54.5	45.5
At C5	102	42	9	48	201	144	57	71.6	28.4	111	90	55.2	44.8
At F2	87	45	14	54	200	132	68	66	34	101	99	50.5	49.5
At F4	62	76	24	38	200	138	62	69	31	86	114	43	57
At G6	79	41	22	58	200	120	80	60	40	101	99	50.5	49.5

Sample	wt+	wt-	daz+	daz-	Total	WT + %	WT - %	daz + %	daz - %	WT %	daz %	RFP+	%	RFP-	%
Sl A6	68	36	28	45	177	38	20	16	25	58.8	41	96	54	81	46
Sl B5	40	40	17	43	140	29	29	12	31	57.1	43	57	41	83	59
Sl C2	35	41	24	35	135	26	30	18	26	56.3	44	59	44	76	56

Sample	wt+	wt-	daz+	daz-	Total	WT + %	WT - %	daz + %	daz - %	WT %	daz %	RFP+	%	RFP-	%
Brapa A2	68	36	25	49	178	38	20	14	28	58.4	42	93	52	85	48
Brapa A4	54	25	28	30	137	39	18	20	22	57.7	42	82	60	55	40
Brapa B4	63	36	27	47	173	36	21	16	27	57.2	43	90	52	83	48
Brapa D4	72	50	35	59	216	33	23	16	27	56.5	44	107	50	109	50

Table A9: Frequency of the rescue of male transmission in selected species DAZ1

AtDAZ1		**Resistant**	**Sensitive**	**Ng**	**Total**	**Sum**	**%R**
C5 line B3	91	47	22	160	138	65.9	**%S**
C5 Line B6	103	51	6	160	154	66.9	34.1
D5 Line C4	64	36	60	160	100	64.0	33.1
D5 Line C6	84	43	33	160	127	66.1	36.0
Total	342	177		640	519		33.9

SlDAZ1	**Resistant**	**Sensitive**	**Ng**		**R/S**	**% R**	
A6	49	43		92	1.14	53.26	**%S**
B5	38	28		66	1.36	57.58	46.74
Total	87	71		158	1.23	65.74	42.42
							34.26

BrDAZ1	**Resistant**	**Sensitive**	**Ng**	**total**	**Sum**	**%R**	
A2	45	39		84	1.15	53.57	**%S**
B4	57	43		100	1.33	57.00	46.43
Total	102	82		184	1.2	58.86	43.00

Table A10; Measurement of the species triple homozygous DAZ 1 siliques lengths

Columbia (Wildtype)

line	silique	length (cm)	line	silique	length (cm)	line	silique	length (cm)
A1	1	1.7	**A2**	1	1.7	**A3**	1	1.6
	2	1.8		2	1.7		2	1.7
	3	1.7		3	1.6		3	1.8
	4	1.8		4	1.8		4	1.7
	5	1.7		5	1.8		5	1.8
	6	1.9		6	1.7		6	1.6
	7	1.8		7	1.8		7	1.7
	8	1.7		8	1.6		8	1.8
	9	1.8		9	1.7		9	1.7
	10	1.7		10	1.6		10	1.7
	MEAN	**1.76**		**MEAN**	**1.7**		**MEAN**	**1.71**

daz1-1 -/- daz2-1 +/-

line	silique	length (cm)	line	silique	length (cm)	line	silique	length (cm)
A1	1	1.1	**A2**	1	1.1	**A4**	1	1.1
	2	1.2		2	1.3		2	1
	3	1.1		3	1.1		3	1.1
	4	1.1		4	0.8		4	0.9
	5	1.1		5	1.3		5	0.9
	6	1.3		6	1		6	1.3
	7	1.1		7	1.2		7	1.2
	8	1.1		8	1.1		8	1
	9	0.7		9	0.9		9	1.1
	10	0.7		10	0.9		10	1.2
	MEAN	**1.05**		**MEAN**	**1.07**		**MEAN**	**1.08**

Arabiopsis thailana

line	silique	length (cm)	line	silique	length (cm)	line	silique	length (cm)
D6	1	1.5	**B3**	1	1.7	**C3**	1	1.6
	2	1.5		2	1.7		2	1.6
	3	1.5		3	1.6		3	1.5
	4	1.5		4	1.7		4	1.7
	5	1.6		5	1.8		5	1.5
	6	1.5		6	1.7		6	1.6
	7	1.6		7	1.6		7	1.7
	8	1.5		8	1.6		8	1.8
	9	1.6		9	1.7		9	1.5
	10	1.5		10	1.6		10	1.5
	MEAN	**1.53**		**MEAN**	**1.67**		**MEAN**	**1.6**

Solanum lycopersicum

line	silique	length (cm)	line	silique	length (cm)	line	silique	length (cm)
D6	1	1.3	**D1**	1	1.3	**D3**	1	1
	2	1.1		2	1.3		2	0.9
	3	1.3		3	1.1		3	1.1
	4	1.2		4	1.1		4	1.1
	5	1.3		5	1.2		5	1.3
	6	1.1		6	1.4		6	1.5
	7	1.4		7	1.3		7	1.6
	8	1.4		8	1.2		8	1.5
	9	1.2		9	1.2		9	1.4
	10	1.3		10	1.4		10	1.5
	MEAN	**1.26**		**MEAN**	**1.25**		**MEAN**	**1.29**

Brassica rapa

line	silique	length (cm)	line	silique	length (cm)	line	silique	length (cm)
D6	1	1.2	**D1**	1	1.3	**D3**	1	1.3
	2	1.2		2	1.3		2	1.1
	3	1.3		3	1.1		3	1
	4	1.2		4	1.2		4	1.2
	5	1.3		5	1.1		5	1.2
	6	1.2		6	1.2		6	1.3
	7	1.3		7	1.3		7	1.3
	8	1.2		8	1.2		8	1.3
	9	1.1		9	1.2		9	1.1
	10	1.2		10	1.3		10	1.2
	MEAN	1.22		**MEAN**	1.22		**MEAN**	1.2

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