Genetic analysis of sperm cell formation in
*Arabidopsis thaliana* L. Heynh.

Thesis submitted for the degree of
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by

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For Papa, Mama
&
Ramesh
ABSTRACT

Genetic analysis of sperm cell formation
in Arabidopsis thaliana L. Heynh.

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The genetics and cell biology of a novel class of mutants that affect microgametogenesis in Arabidopsis was investigated. These mutants termed duo pollen (duo) specifically block generative cell division preventing the formation of sperm cells. Genetic analyses revealed that duo1-duo6 act gametophytically and are male-specific. The duo mutants mapped to different chromosomal locations. Phenotypic characterisation revealed that duo1-duo3 share similar but distinct nuclear morphologies. Analysis of pollen grains exhibiting mitotic figures showed that the undivided generative cell in duo1 entered pollen mitosis II but failed to progress beyond prometaphase. In contrast duo2 and duo3 failed at G2-M transition of pollen mitosis II. The progression of S phase was monitored by measuring nuclear DNA contents throughout gametogenesis in the wild type and in duo1-duo3. In the wild type, sperm nuclei enter S phase soon after inception and continue DNA synthesis until anthesis. Moreover, the male gametic cells in wild type appear to follow a simple S to M cycle. In duo1 the generative nucleus completes DNA synthesis and maintains a 2C DNA value at anthesis. In contrast, mutant generative nuclei in duo2 and duo3 bypass pollen mitosis II and continue DNA synthesis during pollen maturation similar to sperm nuclei in the wild type. Map-based cloning defined the DU03 locus to a 10 kb region. One of the genes, termed DU03 successfully complemented the duo3 mutation. Nucleotide sequencing of DU03 in heterozygous duo3 mutants revealed no sequence differences from the wild type, suggesting that DU03 is an epiallele. DU03 encodes a novel protein of 137 kDa containing a Myb-like domain known as the SANT domain. This work demonstrated gametophytic control that operates at G2-M and M phase of generative cell cycle. The identification of DU03 gene provides a unique opportunity to understand the molecular basis of the generative cell division.
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### Abbreviations

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<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>°C</td>
<td>degrees centigrade</td>
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<tr>
<td>cM</td>
<td>centimorgan</td>
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<tr>
<td>cm</td>
<td>centimetre</td>
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<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DW</td>
<td>distilled water</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
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<tr>
<td>kb</td>
<td>kilobase pair</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
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<td>l</td>
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<tr>
<td>M</td>
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<tr>
<td>MES</td>
<td>2-(N-Morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>Mts</td>
<td>Microtubules</td>
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<td>µg</td>
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<td>MS</td>
<td>Murashige and Skoog</td>
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<td>ng</td>
<td>nanogram</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PNAACL</td>
<td>The protein and nucleic acid chemistry laboratory</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>rpm</td>
<td>revolution per minute</td>
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<td>room temperature</td>
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<td>RT-PCR</td>
<td>reverse transcriptase-PCR</td>
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<td>standard deviation</td>
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<tr>
<td>SSLP</td>
<td>simple sequence length polymorphisms</td>
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<tr>
<td>TAE</td>
<td>tris-acetate EDTA</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
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<tr>
<td>Triton X-100</td>
<td>(t-Octylphenoxypolyethoxyethanol)</td>
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<td>U</td>
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<td>wild type</td>
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<td>weight per volume</td>
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Chapter One
Introduction
Chapter One
Introduction

1.1 Life cycle of plants

Sexual reproduction in the plant kingdom has a selective advantage over asexual reproduction as it enhances the crucial recombination events that increase the plasticity within a species and promote evolution. Flowering plants constitute the largest group within the plant kingdom and dispersal of flowering plants by seeds has contributed to the expansion of this group of plant species. Plant life cycles alternate the growth of the diploid sporophytic organism with the growth of another form, the haploid gametophyte. In angiosperms the growth and development of the gametophyte is reduced, but the developmental programs are still complex. In flowering plants pollen grain represents the male gametophyte.

In addition to its vital role in sexual reproduction, pollen offers an excellent system to study developmental processes such as cell division as it has a consistent and simple cell lineage that is relatively easy to follow compared with lineages that derive most other plant structures (Bedinger, 1992; McCormick, 1993; Twell, 1994, Twell, 2001).

1.2 Pollen cell lineages and development

Male gametophyte development takes place within the anther, as shown in Figure 1.1, starting with the formation of archesporial cells in the anther primordia. Divisions of the archesporial cells results in a primary parietal layer and an inner sporogenous layer. The cells of the primary sporogenous layer undergo mitosis to give rise to diploid meiocytes or pollen mother cells (PMCs). PMCs undergo meiosis to give rise to a tetrad of four haploid microspores that are encased in a callose (β-1,3-glucan) wall. Completion of meiosis of the microsporocyte and the initial burst of growth and exine synthesis
Figure 1.1: Schematic representation of pollen developmental pathway.
marks the initiation of a unique cellular differentiation, microgametogenesis that leads to the formation of mature pollen grains through a simple cell lineage. Upon degeneration of the callose wall, the haploid microspore leads an independent development, permitting the haploid genome to function independently of the diploid parent (Knox, 1984, Mascarenhas, 1990, Bedinger, 1992, Twell, 1994). In the Arabidopsis quartet mutants, *qrt1* and *qrt2*, the microspores remain attached in a tetrad at anthesis (Preuss et al., 1994). *QRT1* and *QRT2* appear to function in this cell type specific removal of pectin (Rhee and Somerville, 1998). Vacuoles play an important role in the development of the microspore. Rapid expansion of the microspore is achieved by the vacuole growth. Newly released microspore contains clusters of small vacuoles that rapidly increase in size and number. By mid-microspore development the vacuoles enlarge and fuse to create several larger vacuoles. The vacuoles fuse to produce a single large vacuole that occupies major part of the cell during late microspore development. This large vacuole displaces the microspore nucleus to an eccentric position against the wall (Owen and Makaroff, 1995).

### 1.3 Formaion of the male germ line following asymmetric division

Each uninucleate microspore undergoes an asymmetric mitotic division to give rise to two cells with distinct cell fates. Asymmetric division at PMI marks the end of microspore development and the initiation of pollen development. A preprophase band (PPB) of microtubules marks the future division plane and the site of cytokinesis in somatic cells. The cell plate arises from the phragmoplast in the centre of the division plane and grows centrifugally towards the parental cell wall (Heese et al., 1998; Sylvester, 2000). In gametophytic cytokinesis at PMI, the preprophase band is absent and a unique hemispherical cell plate is formed that is curved around the eccentric generative nucleus (Van Lammeren et al., 1985; Terasaka and Niitsu, 1995, Tanaka 1996, Gunning and Steer 1996). Curved profiles of the phragmoplast microtubules appear to guide the centrifugal growth of the cell plate at its margins to ensure asymmetric cytoplasmic cleavage (Brown and Lemmon, 1994; Terasaka and Niitsu, 1995). In moth orchids (*Phalaenopsis sp.*) unequal first mitosis in pollen is associated with a generative-pole microtubule system (GPMS) (Brown and Lemmon, 1991a, b).
But in species like *Tradescantia paludosa*, *Cypripedium fasciculatum* and *Brassica napus* the GPMS have not been found in pollen mitosis (Terasaka and Niitsu 1990, Brown and Lemmon 1994, Hause et al., 1992). The intrinsically asymmetric division at PMI results into two daughter cells with differential cell fate (Twell et al., 1998). The larger vegetative cell shows disperse nuclear chromatin and contains most of the pollen cytoplasm whereas the smaller lens shaped generative cell shows condensed nuclear chromatin and contains relatively few organelles and stored metabolites. The vegetative cell exits the cell cycle and remains arrested in G1 (Zarsky et al., 1992). The generative cell represents the male germ line in flowering plants and has the ability to undergo a further mitotic division to form two sperm cells.

### 1.4 Detachment and migration of the generative cell

A uniquely specialised cell-cell co-operation leads to generative cell migration that culminates into 'cell within a cell' structure. The asymmetric division at PMI partitions the GC from the VC by a hemispherical callose wall. This callose wall is lost while the GC is still attached to the intine. As the wall degrades the GC detaches from the intine wall layer and it moves inward. The GC pushes forward into the vegetative cell, a constriction is formed between the generative nucleus and the intine extends inwards. The connection is interrupted and the GC becomes completely engulfed by the vegetative cell (Sanger and Jackson, 1971a). In pollen grains of *Polystachia pubescens* (Orchidaceae) plasma filled vesicles line the original contact area of the GC and the intine. During the detachment the vesicles are left behind by the generative cell, probably due to the dynamic nature of the detachment process the plasma membrane of the GC repeatedly tears at the margin of the base and is sealed only when it is engulfed (Schlag and Hesse, 1992). The driving force behind the detachment process of the GC is poorly understood. However, microtubules arrays are strongly associated with the internal membrane of the GC and the vegetative nucleus immediately after PMI suggesting that the cytoskeleton is implicated in generative cell migration (Zonia et al., 1999).
1.5 Generative cell morphogenesis and the distribution of microtubules

The lens shaped generative cell undergoes change in shape after detachment from the pollen wall. The GC rounds up to acquire a spherical shape during the process of engulfment into the vegetative cytoplasm. Prior to generative division the GC undergoes further morphogenesis and acquires an elongated lenticular or spindle shape (Palevitz and Tiezzi, 1992). Generative cell morphogenesis and the detachment of the generative cell from the inner pollen grain are associated with microtubules (Mts) array (Heslop-Harrison, 1968). A prominent feature of the generative cell is the basket-like arrays of cross-bridged Mts aligned helically or longitudinally relative to the long axis (Heslop-Harrison et al., 1988)

The significance of the axial Mts bundles in maintaining GC shape has been demonstrated in the male gametic cells. It has been shown that microtubule-perturbing drugs prevent the elongation of the generative cell from spherical to spindle-shaped (Sanger and Jackson, 1971a). Generative cells in colchicine-treated pollen round up and fail to undergo the usual change in shape during passage through the pollen tube (Heslop-Harrison et al., 1988). The spindle-shaped generative cells in lily and sperm cells in Spinacia oleracea revert to a spherical shape after liberation (Tanaka et al., 1989; Theunis, 1990). Establishment or maintenance of cell shape, morphogenesis and the participation in the generative cell division are the functions most commonly assigned to the Mts (Palevitz and Tiezzi, 1992). However, microtubule arrays are not sufficient to maintain cell shape in isolated GC, unknown interactions between the GC surface and the VC cytoplasm may exist in maintaining the generative cell and the sperm cell shape.

1.6 Significance of PMI

The asymmetric division of microspore at PMI culminates in the production of two daughter cells with differential cell fates. Inhibition of asymmetric cell division to symmetric division by microtubule-perturbing drug prevents differentiation of the generative cell and a cell-within-a-cell organisation is not formed (Bishop and
McGowan, 1953; Tanaka and Ito 1981; Eady et al., 1995). The results directly demonstrated that asymmetric division at PMI is essential for correct GC differentiation. The exact mechanisms by which the generative cell differentiation occurs remains unknown, however two models have been formulated for the generative and vegetative cell differentiation. In the passive model the asymmetric division leads to the repression of genes in the generative cell and activation of the vegetative cell genes due to the passive exclusion of the gametophytic factors from the generative cell pole. In the active model, a generative cell repressor is localised in the generative cell pole and it blocks the action of the gametophytic factor (Eady et al., 1995).

1.7 Organelles in the generative cell and its dependence on the vegetative cell

Plastids are excluded from the generative cell during the first pollen mitosis and are passed on to the vegetative cell. Extranuclear organelle DNA is inherited maternally in the majority of angiosperms. The mitochondrial DNA in the male reproductive cells of *Hordeum vulgare* was examined by immunoelectron microscopy. The results showed that the number of anti-DNA gold particles on the sections of sperm cells mitochondria decreased by 97% during pollen development. The reduction occurred rapidly in the generative cells and subsequently in the sperm cells (Sodmergen et al., 2002).

The generative cell remains suspended in the vegetative cell that provides a specialised environment for its support and nutrition (Chaboud and Perez, 1992; Palevitz and Tiezzi, 1992). Evidence for the dependence of the GC on the VC is provided by targeted cell ablation in which the vegetative cell was ablated, by the expression of the cytotoxic diphtheria toxin A chain (DTA), under the control of the vegetative cell-specific LAT52 promoter (Twell, 1995). Expression of the LAT52-DTA caused the rapid loss of the membrane integrity and death of the VC. The GC failed to move into the degenerating VC, showing the dependence of migration on the presence of an intact VC.
1.8 Generative cell division

The generative cell represents the male germ line, the progenitor of the two sperm cells in flowering plants. The development of the male gametophyte follows one of the two highly conserved pathways in angiosperm plants. Out of 2000 flowering plants studied approximately 70% of plants species shed bicellular pollen grains and mitosis of the generative cell subsequently occurs within the growing pollen tube following germination. The remainder of plant species shed tricellular pollen grains such that generative cell division is activated and completed before anthesis. All the phylogenetically primitive taxa are bicellular and the tricellular trait originated independently at many times during angiosperm evolution from the pleisomorphic bicellular pollen. This heterochronic shift in the timing of generative cell division is presumably the outcome of an adaptive evolution in angiosperms (Brewbaker, 1967).

1.8.1 Prophase and prometaphase

In tobacco prior to mitosis the GC has a highly elongated structure and chromatin is condensed in the nucleus. At prophase, chromatin undergoes further condensation. The number of cytoplasmic Mts decreases and disappears completely by late prometaphase however there is an increase of Mts in the nuclear region. The beginning of prometaphase is marked by the breakdown of nuclear envelope (Yu and Russell, 1993). In *Ornithogalum virens*, the three chromosomes are highly condensed at prophase and the cytoplasmic Mts are organised into three thick bundles and remain spatially related to the position of the generative nucleus. At prometaphase Mt bundles diffuse into the nuclear area and the spindle fibres originate from the prophase Mt bundles. At prometaphase the nuclear membrane breaks down and a rapid rearrangement of the Mts occurs (Charzynska and Cresti, 1993; Banas et al., 1996).

1.8.2 Metaphase

The distribution of kinetochores at metaphase during GC division in pollen tubes has been the subject of conflicting reports. In species like, *Hyacinthus* the kinetochores are
conventionally distributed along the equatorial plane (Del Casino et al., 1992). In *Ornithogalum*, metaphase of the GC occurs as in the somatic cells and the kinetochores are located in one plane perpendicular to the pole of the spindle axis (Banas et al., 1996). In species like *Rhododendron* and *Tradescantia* chromosomes are arranged perpendicular to the oblique spindle with respect to the equatorial plane (Taylor et al., 1989; Lui and Palevitz, 1991; Palevitz, 1990; Palevitz and Cresti, 1989). In tobacco, the spindle is characterised by the longitudinal distribution of the kinetochores, perpendicular spindles and a highly dispersed spindle poles. Three-dimensional reconstruction data on style-grown pollen tubes of *Nicotiana tabacum* revealed a mitotic apparatus in which kinetochores are irregularly distributed along the length and depth of the GC and are not strictly planar in distribution (Yu and Russell, 1993).

### 1.8.3 Anaphase

In *Tradescantia*, anaphase of the GC consists of three main events. At the onset of anaphase, two superbundles are formed from opposite poles of the kinetochore fibers and associated Mts, the next step involves the shortening of kinetochores and then separation of superbundles and mitotic poles (Lui and Palevitz, 1991). However, in tobacco the superbundles have not been reported, but the movement of the chromosomes seems to result from the convergence of sub-poles into pointed poles, shortening of the kinetochores bundles and the separation of anaphase poles (Yu and Russell, 1993).

### 1.8.4 Telophase and cytokinesis in the GC

In tobacco, cytokinesis appears conventional. Cell plate is formed at the equatorial plane resulting in the formation of isomorphic sperm cells (Yu and Russell, 1993). In *Brassica napus*, cytokinesis occurs by two concerted actions, the generative cell becomes constricted to form a dumbbell structure and coalescence of the golgi vesicles leads to cell plate formation at the equatorial plane (Murgia et al., 1991). Whether newly formed sperm cells separate by means of a conventional cell plate formation with the participation of the phragmoplast or by cellular constriction similar to midbody
cleavage in animals seems to be a controversy. In species like Alocasia, Lilium, Nicotiana and Rhododendron, a conventional phragmoplast is formed during sperm cell formation. However, species like Tradescantia and Hyacinthus appear to lack a phragmoplast or a cell plate (Del Casino et al., 1992; Palevitz and Cresti, 1989). Cytokinesis during generative cell division in Tradescantia seems to involve cellular constriction, however a circumferential ring of F-actin might not be involved. Although initially controversial, the weight of evidence has demonstrated the absence of F-actin in the generative and sperm cell, excluding a role in GC division (Palevitz and Liu, 1992).

1.9 Gene regulation during pollen development

Pollen development requires the coordinated control of genes expressed both in the gametophytic and sporophytic generations (McCormick, 1991; Scott et al., 1991; Bedinger, 1992). Microgametogenesis comprises two distinct developmental phases: microspore development that terminates with the completion of PMI and pollen development that culminates in the production of mature pollen grains. Isolated genes that are specifically or preferentially expressed in the male gametophyte are therefore classified into two groups, the early genes and the late genes (Scott et al., 1991, Twell et al., 1989). The late genes appear to be activated in close association with or soon after microspore mitosis and their transcripts accumulate until maturity. In contrast, the early genes are activated before PMI, that is, in the tetrad or young microspore and their transcript decline in abundance before pollen maturation.

There is relatively little information about the specific genes activated in the microspore. The regulation of gene expression during pollen development has been analysed for a number of anther-specific genes by determining the kinetics of transcripts and the proteins (McCormick, 1993 and Mascarenhas, 1992). Anther-specific genes expressed during the early stages of pollen development were identified and characterised on the basis of sequence homology and mostly encode proteins of tapetal origin. Other genes encoding proteins involved in pollen exine formation, in lipid transfer and cell wall proteins have also been identified in the early stages of male
gametophyte development (Goldberg et al., 1993). Northern blot analysis revealed two distinct classes of anther-specific genes (Bp4 and Bp19) from *Brassica napus* that are expressed in developing microspores (Albani et al., 1990, 1991). An *Arabidopsis* anther-specific gene (*apg*) directs β-glucuronidase (GUS) activity in both the sporophyte and the gametophyte (Roberts et al., 1993). Developmental regulation of the *apg* promoter in the transgenic tobacco shows that *apg* promoter is activated in a biphasic pattern (Twell et al., 1993). The anther-specific gene, *Bcp1*, from *Brassica campestris* has a unique pattern of expression in the tapetum as well as in the microspore (Theerakulpisut et al., 1991). Similarly, in *Arabidopsis* the gene *Bcp1* is active in both diploid tapetum and haploid microspore and is required for pollen fertility (Xu et al., 1995). In tobacco, the transcripts of *NTM19* are detected after microspore release and are rapidly degraded before PMI (Oldenhof et al., 1996). *TAZ1* (tapetum development zinc finger protein) cDNA was isolated as an anther-specific cDNA from petunia. Spatial and temporal expression profiles revealed that *TAZ1* showed a biphasic expression pattern and was demonstrated to play an essential role in the post-meiotic phase of tapetum development (Kapoor et al., 2002).

Another level of control exits to regulate the cell-specific expression of late pollen genes between the vegetative and the generative cells. Evidence for vegetative cell-specific regulation of late pollen genes was obtained by linking the promoter of the tomato *LAT52* to a nuclear-targeted GUS fusion protein gene. *Lat52* promoter was activated specifically in the vegetative nucleus (Twell, 1992).

Recent microarray technology has demonstrated the diversity of the haploid genome in *Arabidopsis thaliana* (Honys and Twell, 2003, Becker et al., 2003). Mature pollen grains express over 5,000 different mRNA out of the 27,000 predicted from the *Arabidopsis* genome. Approximately 40% of these transcripts are predicted to be specifically or preferentially expressed in the male gametophyte. There is a significant overlap between sporophytic and gametophytic gene expression that indicates the large pool of genes that are required for basic cellular processes. The transcriptome analysis reveals that the most abundant classes of pollen-specific genes are associated with
transcriptional regulation, signal transduction, cytoskeleton organisation and cell wall synthesis (Honys and Twell, 2003).

1.10 Gene expression in the generative cell and the sperm cell

Due to the more condensed state of the generative cell, it was presumed that the generative nucleus is transcriptionally quiescent and the vegetative nucleus is more active (McCormick, 1993). Important development was made to isolate and culture male gametic cells in maize and lily (Zhang et al., 1993; Ueda and Tanaka, 1995). However, the number of genes identified in the generative cell is limited.

It was demonstrated by metabolic labelling with $^{35}$S-methionine that the generative cells of *Lilium longiflorum* possess their own set of mRNA and are capable of synthesising proteins independently from the vegetative cell. The isolated generative cells synthesised ten proteins of which six were unique to the specialised cells (Blomstedt et al., 1996). *LCG1* was isolated from Lily CDNA library and the expression was localised exclusively in the male gametic cells. It was predicted that LGC1 protein was associated with the plasma membrane of the generative cell and hence play a role in cell-to-cell recognition. Northern hybridisation and reverse transcription-polymerase chain reaction (RT-PCR) confirmed the generative cell specificity of this clone (Xu et al., 1999a). In addition, the diphtheria toxin A-chain- (DT-A)-coding region under the control of the *LGC1* promoter confirmed the lack of *LGC1* expression in the vegetative tissues. Regulatory sequences required for determining generative cell-specific expression of *LGC1* was identified. Deletion of this regulatory sequence led to loss of the generative cell specificity resulting in activation of this promoter in other tissues (Singh et al., 2003). A homolog of a human excision repair has also been isolated from gametic cells and designated as *ERCC1* gene. The gene is preferentially expressed in the male gametic cells and presumed to protect the germ-line DNA from environment agents such as UV radiation (Xu et al., 1998). RT-PCR analysis revealed the expression of cyclin-dependent kinase, cyclin A1 and histone H3 in sperm cells (Sauter et al., 1998).
To clone novel genes that are expressed specifically in male gametic cells, a differential-display method with total RNA was applied to isolated generative cells of *Lilium longiflorum* and at least three fragments were specifically amplified in the generative cells compared with the pollen and the leaf cells. Northern blot analysis showed that one of these, termed AP6-E, was part of a gene expressed strongly in generative cells. The isolation of AP6-E cDNA including an open reading frame of 468 amino acids showed that the AP6-E protein is a homolog of the plant plastid division protein *ftsZ*. The generative cell of *L. longiflorum* lacks plastids, the expression of the plant plastid division protein in these cells was contradictory. Two hypotheses were put forward to explain this contradiction, the LIFtsZ gene is only transcribed in the generative cells and the protein might not be functional or the LIFtsZ protein has some unknown unrelated to plastid division (Mori and Tanaka, 2000). A plant ortholog of the *glsA* gene was isolated from *L. longiflorum* generative cells. GlsA is a chaperone-like protein essential for gonidia production and was identified in an asexual-reproductive-cell deficient mutant of *volvox*. Immunoblot analyses revealed that strong *LIGlsA* expression occurs preferentially in the generative cells (Mori et al., 2003).

Highly pure sperm of *Zea mays* was obtained by fluorescence-activated cell sorted (FACS) and a high-quality cDNA library was constructed. Sequencing of over 1100 cDNAs from the amplified library revealed that sperm have a diverse complement of mRNAs, about 8% of the sequences are predicted to encode plasma membrane-localised proteins and predicted to play a role in gamete interactions. Sperm transcripts encoding proteins involved in general cell function were present throughout pollen development and were more abundant in tricellular pollen than in sperm cells. Several transcripts encoding proteins that are similar to hypothetical *Arabidopsis* proteins appeared exclusively in the sperm cells in mature pollen and unicellular microspores, suggesting that certain transcripts are transcribed early during pollen development and are subsequently separated into sperm cells (Engel et al., 2003).
1.11 Generative cell specific histones

A characteristic feature of angiosperm male gametic cells (generative cell and sperm cell) is that they have highly condensed chromatin as the motile sperms of animals and lower plants (McCormick, 1991; Tanaka, 1997). The composition of the nuclear chromatin is not very clear. Two-dimensional gel electrophoresis of basic proteins extracted from the isolated generative and vegetative protoplast revealed two proteins resembling HI histones and three other resembling core histones. The transcript of the three male gamete-specific histones gH2A, gH2B and gH3 were detected only in the cytoplasm of the generative cell. It was predicted that the male gamete-specific histones in *Lilium* play a homologous role to the protamine of mammalian sperm in chromatin remodelling of male gametes, and result in complete suppression of gene expression in the male gametes (Ueda and Tanaka, 1994).

Histones or histone-like proteins appear to be associated with chromatin formation at the most fundamental level of chromatin structure, the nucleosome. Using specific antibodies raised against gH2B and gH3, the temporal and spatial aspects of the appearance of these histones were examined. It was found that neither gH2B nor gH3 was detected in the microsporocytes during meiotic division or in microspores before microspore mitosis. Both histones were present abundantly in mid bicellular pollen and they continued to be detected in germinated pollen tubes after pollination. Moreover, immunocytochemistry revealed that these proteins were present in sperm nuclei. It was speculated that the histones might be newly synthesised in the sperm cell for differentiation of the male gametes (Ueda and Tanaka, 1995).

The chromatin in the vegetative nucleus gradually disperses after PMI, whereas the chromatin in the generative nucleus remains highly condensed. To understand the cause of this difference, histone composition was analysed immunocytochemically in each nucleus in *Lilium longiflorum* and *Tulipa gesneriana*. Specific antisera were raised against histones H1 and H2B of *Lilium*. The results showed that the level of histones H1 gradually decreased only in the vegetative nucleus during the development of the pollen development and was greatly reduced at anthesis. The amount of histone H2B in both
nuclei was the same. Moreover, preferential decrease in the level of histone H1 occurred in colchicines induced uninucleate and in equally divided pollen (two similar nuclei). This showed that preferential decrease in the level of histone H1 is essential for the development of the male gametophytic cell (Tanaka et al., 1998). Two clones encoding generative cell-specific histones, designated gcH2A and gcH3 were obtained from a cDNA library of isolated generative cells in Liliium. The transcripts of both gcH2A and gcH3 increased progressively during generative cell maturation and were presumed to play a role in maintaining the condensed state of chromatin in the generative cell (Xu et al., 1999b)

1.12 Methylation of the male genome

DNA methylation represents an important factor that plays a long-term control role of gene expression. DNA methylation stabilises the condensed chromatin structure, presumably via binding of specific proteins (Kass et al., 1997). Oakeley et al. (1997) showed that DNA methylation level in the vegetative nucleus of tobacco is higher than that in the generative nucleus. H4 acetylation and DNA methylation was analysed in microspores, immature binucleate pollen, mature pollen and pollen tubes in Liliium longiflorum. The result shows that histone H4 of the vegetative nucleus inspite of its decondensed chromatin structure is strongly hypoacetylated compared to microspore and generative nucleus. During pollen tube growth the H4 terminal lysine in the vegetative nucleus gradually becomes acetylated. However, the DNA methylation status inversely correlates with the histone acetylation data and the vegetative nucleus in the mature pollen grains is heavily methylated during the pollen tube development. In marked contrast, H4 acetylation and DNA methylation status remain unaltered during development of the generative nucleus (Janousek et al., 2000).

1.13 Pollen as a model to study cell cycle patterns

Pollen grains of flowering plants represent the haploid partner in sexual reproduction and their role is to deliver the two sperms cells, via the pollen tube to the embryo sac to effect double fertilisation. Variability in the cell cycle patterns associated with the male
gametes maturation can be followed. During sexual reproduction male and female gametes coordinate ontogenic events associated with gametogenesis and successful fertilisation through a key regulator, the cell cycle.

### 1.14 Cell cycle in the gametophyte

A number of cellular processes such as cell division, cell growth and differentiation are modulated by the activities of the cell cycle (Soni et al., 1995; Hirt, 1996; Shaul et al., 1996; Umeda et al., 2000). Evidence for cell cycle regulation during microspore development comes from the division mutants, Gemini pollen mutant and Sidecar pollen mutant (Chen and McCormick, 1996; Park et al., 1998). The male gametophytic mutant Sidecar pollen (scp) mutant shows premature symmetric division of the microspore and the two daughter cells follow different fates. One of them follows vegetative cell fate and the other daughter cell follows normal microspore program by establishing polarity and asymmetric division. The Gemini pollen (gem) mutant also shows symmetric division at PMI, but the daughter cells do not express polarity and both cells follow the vegetative cell fate. These mutants highlight the importance of coordinating the microspore cell cycle with the development of cytoplasmic polarity to achieve asymmetric division and the formation of correct number of cells in the mature pollen grain.

Among plants male and female gametes coordinate ontogenic events associated with gametogenesis and fertilisation through the cell cycle. Successful fusion of gametes in plants depends upon the sperm and the egg reaching synchronous position within the cell cycle prior to fertilisation and during karyogamy. The male gametophyte of Ephedra trifurca sheds bicellular pollen grains, pollen mitosis II is completed in the pollen tube and the sperm nuclei remains in G1 during their passage through the pollen tube to the egg cell. Upon entry into the egg cell, the sperm nuclei pass into synthesis phase of the cell cycle. A prolonged contact between the male and the female nuclei exists during sexual reproduction in Ephedra presumably to allow the gametes to complete S phase. Complete fusion of the male and female gamete nuclei occurs when the DNA content reaches 2C level (Friedman, 1991). Gnetum gnenom, a non-flowering
seed plant expresses a rudimentary pattern of double fertilisation resulting in the formation of two zygotes per pollen tube. The generative nucleus is haploid and is predicted to pass through the S phase of the cell cycle before its mitotic division. Newly formed sperm nuclei contain 1C quantity of DNA and do not remain in the G1 phase for an extended period but enter S phase of the cell cycle soon after its formation. By the time the pollen tube enters the female gametophyte the sperm nuclei contains 2C quantity of DNA. Thus, in Gnetum gnenom the male gametes complete DNA replication before the onset of fertilisation. The 2C male gametes are discharged into a coenocytic female gametophyte that lacks defined egg cells. The release of the two sperm nuclei from the pollen tube and fusion with the female nucleus appear correlated with gamete nuclei attaining a precise stage within the cell cycle (Carmichael and Friedman, 1995).

In Arabidopsis, the relationship between developmental events and the cell cycle in sperm cells were examined. Newly formed sperm nuclei rapidly enter S phase of the cell cycle and continue to synthesise DNA such that at pollination the DNA content reaches 1.5C. Following pollination, sperm nuclei continue through S phase of the cell cycle during pollen tube. By the time the pollen tube reaches the ovary, the sperm nuclei contains approximately 1.75C DNA and prior to fertilisation the DNA content attains 2C. These data clearly demonstrated that the molecular programs associated with S phase of the cell cycle are expressed in the sperm cells (Friedman, 1999).

Comparative ontogenetic sequence analysis revealed that there are five different and basic patterns of sperm development among seed plants. The pattern of sperm and male gametophyte development are based on the relative timing and expression of the sperm cell cycle. In bicellular species such as Ephedra and Tradescantia both sperm cells retain 1C content of DNA throughout the development of pollen tube, the bicellular-G1 model of development. In Gnetum, sperm initiates and completes DNA synthesis within the pollen tube, thus considered as the bicellular-G2 type. Among tricellular species, Zea, Hordeum and Dendranthema, the sperm cells retain 1C level of DNA content and this ontogeny is described as the tricellular-G1. In Arabidopsis, S phase of the sperm cell cycle is initiated in mature pollen and completed in the pollen tube, indicating a tricellular-G2 type of development. In the taxa, Crepis and Elytrigria, S phase of the
cell cycle is completed prior to pollen maturation and this ontogeny is described as tricellular-G2 precocious (Friedman, 1999).

1.15 Cell cycle controls in plants

Coordinated cell cycle progression of the male and female gametes is a prerequisite for successful sexual reproduction. In higher plants, the cell cycle progression of the male gametes is quite variable and remains arrested at either G1 or G2 of the cell cycle (Friedman, 1999). The basic mechanism of cell cycle control is apparently conserved in all higher eukaryotes, its regulation can differ according to the different developmental plans and programs of each type of organism (Huntley and Murray, 1999; Potuschak and Doerner, 2001; Stals and Inze, 2001, Criqui and Genschik, 2002).

In eukaryotic cells, initiation of mitosis is governed by a spatial and temporal complex phosphorylation cascade, which leads to the activation of mitosis promoting factor (MPF). MPF consists minimally of the cyclin dependent kinase (CDK) in higher eukaryotes (Cdc2 in yeast) (Ohi and Gould, 1999). In Arabidopsis, three CDK genes have been identified as cdc2a, cdc2b and cdc2c and were also designated as Arath;CDK4;1, Arath;CDKB;1 and Arath;CDKC;1 respectively (Ferreira et al., 1991; Joubes et al., 2000). Proteins such as cyclins, CDK activating kinase (CAK) and CDK inhibitors can regulate the activity of CDK.

Cyclins are well-conserved proteins that are essential for Cdk activation and show periodic accumulation during cell cycle (Mészáros et al., 2000; Stals and Inzé, 2001; Criqui and Genschik, 2002). The Arabidopsis genome contains more than 30 cyclin genes (AGI, 2000). D-type cyclin is thought to play important roles in cell cycle responses to external cues and presumed to play a role in the G1-S phase of transition by cyclin-D-Cdk complexes (Chaboute et al., 2000). A-type cyclin can promote Cdk activity in S and G2 phase. The CycA-Cdk2 complex plays a role in DNA replication during S phase. Multiple A type cyclin exists in plants and they are grouped into three subgroups, A1-A3 (Chaubet-Gigot, 2000). B-type cyclins are expressed specifically in late G2 and early M phase of the cell cycle (Ito, 2000).
In plants, D type cyclins reduce the length of the cell-cycle G1 phase and increases the rate of cell division (Cockcroft et al., 2000). The *Arabidopsis* leaf hairs (trichomes) were used as a model to test the function of the two D-type cyclins, CYCD3;1 and CYCD2;2, in the control of DNA replication and growth in plants. Trichomes are single-celled with polyploid nuclei and ectopic expression of one D-type cyclin, CYCD3;1, induced cell divisions to form multicellular trichomes. The D-type cyclin CYCD3;1 promoted S phase entry and also induced mitosis. These data show that CYCD3;1 expression could shorten the G1 phase and trigger mitosis, raising the possibility that this cyclin represents a novel cell-cycle mode in plants for rapid cycling in response to growth factors (Schnittger et al., 2002).

Full activation of mitotic CDK activity requires not only cyclin association but also an activating phosphorylation on the threonine residue within the CDK T-loop by a CDK-activating kinase (CAK). CDK regulation occurs through the inhibitory phosphorylation of conserved threonine and tyrosines by the kinases myt1 (animals) and wee1 (animals and plants). In *S. pombe*, wee2 is a negative regulator of mitosis and its overexpression causes cells to arrest in G2, to grow but not divide resulting in very elongated cells (Russell and Nurse, 1987). Recently, the rice R2 CAK was found to regulate S-phase progression, however the mitotic functions of CAKs have not yet been demonstrated in plants (Fabien-Marwedel et al., 2002). A functional WEE1 was isolated in *Arabidopsis* by RT-PCR. Semi quantitative RT-PCR showed that *AtWEE1* expression was confined to the dividing regions of the plant (Sorell et al., 2002).

The plant CDK inhibitor was identified in *Arabidopsis* by the yeast two-hybrid technique using *Arabidopsis* cdc2a as bait and the CDK inhibitor ICK1 was shown to inhibit plant CDK kinase activity in vitro (Wang et al., 1997). Six ICK1 related or putative CDK inhibitors were identified in *Arabidopsis* (Lui et al., 2000; De Veyler et al., 2001). Inhibition of plant growth by the expression of a plant CDK inhibitor can be reversed by either down-regulation of the inhibitor gene or by expression of a D-type cyclin that is able to bind to the CDK inhibitor. These observations led to conclusions that CDK inhibitors and the D-type cyclins act in balance to control cell division and
there is a possibility of modifying plant growth and architecture dynamically by adjusting the levels of positive or negative cell cycle regulators (Zhou et al., 2003).

It is established that CDK activities must be switched off during mitotic exit and G1 in order to license replication origins for a novel round of DNA synthesis. Inactivation of CDK occurs essentially through the proteolysis of B-type cyclins by a multisubunit ubiquitin protein-ligase, termed the anaphase-promoting complex (APC/C) (Zachariae and Nasmyth, 1999).

Genome wide analysis is becoming an essential tool to identify and dissect genes involved in controlling biological processes such as cell division (Breyne and Zabeau, 2001). To identify plant genes involved in cell division and controls of cell cycle progression, a genome-wide expression analysis of cell cycle-modulated genes in tobacco Bright Yellow-2 (BY2) was performed. The cDNA-amplified fragment length polymorphism (AFLP) technology was used to identify and characterise a comprehensive collection cell cycle-modulated genes. Approximately 1,340 periodically expressed genes were identified. Once the transcript tags were sequenced, homology searches revealed that 36.5% of the tags were significantly homologous to genes of known functions and 13.1% of the tags matched a genomic sequence without allocated function. For 50.4% of the tags, no homology with a known sequence was found. Interestingly several of the tags homologous to a publicly available sequence have no *Arabidopsis* homolog, indicating that in addition to conserved genes, different plant species possess unique sets of cell cycle-modulated genes (Breyne et al., 2002). Despite the wide range of studies on cell cycle regulation in plants (Menges et al., 2002; Stals and Inze, 2001; Huntley and Murray, 1999; Segers et al., 1996), it remains difficult to understand the cell cycle programs involved during male gametogenesis in *Arabidopsis*. One approach is to create cell division mutants and dissect molecular-genetic mechanisms associated with the cell cycle.
1.16 Screens for gametophytic mutants

A genetic analysis of gametophytic mutants is challenging because not all gametophytic mutations can easily be scored directly for a mutant phenotype nor do they cause sterility. The vast majority of the mutations show a sporophytic requirement and are nuclear recessive, with only a few showing a gametophytic requirement. Deficiency analysis and transmission studies in maize and Arabidopsis reveal that a large number of genes are required during the haploid gametophytic phase (Vizir et al., 1994; Vollbretcht and Hake, 1995).

1.17 Segregation distortion screens

A number of gametophytic mutants affecting various aspects of pollen development and function in Arabidopsis have been identified through genetic screen for segregation distortion (Feldmann et al., 1997, Bonhomme et al., 1998; Howden et al., 1998; Grini et al., 1999). A unique collection of more than 14,000 Arabidopsis transformants was generated with the seed cocultivation method (Feldmann 1991, Feldmann et al., 1994). An initial screening of 142 of these transformed lines on medium containing the selective agent, kanamycin resulted in the identification of 17 lines that failed to segregate the kan^R trait in the Mendelian manner for dominant allele (Feldmann et al., 1997).

T-DNA tagged gametophytic mutants have been identified using T-DNA insertion mutagenesis based on screening for distorted segregation ratios by antibiotic selection. The rationale is that if DNA insertion inactivates an essential gametophytic gene, then the ratio of resistant to sensitive progeny would deviate significantly below the expected 3:1 ratio and would tend towards 1:1 for mutations in male or female specific genes. Approximately 1000 independent Arabidopsis transformants were screened by determination of the segregation of the seedling hygromycin resistance phenotype conferred by the T-DNA. Eight independent putative T-DNA tagged gametophytic mutants affecting male, female or both gametes were identified. These include the cellular morphogenesis mutant, limpet (lip) pollen mutant that fails to complete
generative cell migration, one male specific progamic phase mutant and four lines showing significant effects on male and female transmission (Howden et al., 1998).

The Versailles collection of Arabidopsis T-DNA insertion transformants (Bechtold et al., 1993) was screened for transformants with 1:1 kanR: kanS segregation after selfing, the screen was focused on gametophytic mutations that show normal transmission through one of the gametophyte and cause lethality or disfunction of the other. Only 1.3% (207) out of 16,000 lines screened were recovered as putative candidates. Genetic analysis of 38 putative T-DNA transmission defect lines (Ttd) identified 8 defective gametophytic mutants with reduced transmission through the pollen. Low penetrance mutations affecting both male and female along with many lines with chromosomal rearrangements were recovered from this screen (Bonhomme et al., 1998).

An alternative approach based on the segregation distortion of nearby markers was used to identify gametophytic mutants. This strategy enables the gametophytic mutants to be identified only in close proximity to markers and a multiply marked chromosome I (mm1) that carries five visible recessive markers distributed regularly on the chromosome was used. This strategy allowed simultaneous screening for all the gametophytic mutations induced on this chromosome and EMS was used as a mutagen to induce point mutations. Out of the 200 lines screened, seven gametophytic mutations were isolated that affected the development or function of the pollen or the embryosac. Four mutant lines, mad1, mad2, mad3 and mad4 specifically affected the male gametophyte and the remaining three lines, bod1, bod2, and bod3 affected both the microgametophyte and the megagametophyte. The mad mutants do not enter pollen mitosis II and also display pleiotropic phenotype (Grini et al., 1999).

1.18 Morphological screening of gametophytic mutants

A morphological screen was adopted to identify male gametophytic mutations. Seeds from Arabidopsis, Nossen ecotype (No-0) were bombarded with fast neutron and plants grown from the mutagenized seeds were termed as the M₁ population. Since gametophytic genes are expressed post-meiotically, mutant phenotypes of gametophytes
were screened for and detected in either M₁ sectors or in M₂ plants. Plants showing 50% mutant and 50% wild type were selected. Several mutations with 50% aborted pollen as well as the sidecar pollen (scp) mutant were identified in this screen. The scp mutant acts gametophytically and affects microspore division and cell fate. In the genetic background No-0 ecotype, heterozygous scp plants produce approximately 45% wild type pollen, 48% aborted pollen and 7% pollen with an extra cell (Chen and McCormick, 1996).

The male gametophytic mutant, raging-to-go (rtg) was identified from morphological screen, in which the pollen grains stained for callose before anther dehiscence. Aberrant pollen grains, in rtg plants, acquire or retain water within the anther as early as bicellular stage and germinate. A range of mutants, gift-wrapped pollen (gwp), polka dot pollen (pdp) and emotionally fragile pollen (efp) were obtained from the morphological screening of an M₂ population instead of M₁ population. In the gwp mutants appear to have ribbons tied in bows within the pollen grains. The ribbons appear to be pollen tubes and optical sections through such pollen grains show that the tubes are continuous. In the pdp mutants approximately 50% of the pollen shows brightly stained globules of callose. In somatic cells, callose is formed in response to stress. However, the emotionally fragile pollen mutant was named because even in the absence of stress 50% of the pollen shows diffuse callose staining and sometimes shows more intense staining near the pollen wall (Johnson and McCormick, 2001).

Another screening strategy was adopted to screen for visible mutants that affected the stereotypical pollen cell divisions by examining mature pollen grains after DAPI staining using fluorescence microscopy. Approximately 10,000 M₂ plants from an EMS (Ethyl methanesulfonate) mutagenised population (Nossen background) were screened and 15-20 independently induced mutants were obtained that affected the stereotypical pollen cell division and pollen intracellular architecture (Park et al., 1998).

The gem pollen (gem) mutant that produces twin pollen, the symmetry of division at PMI is affected. In the symmetrically divided cells both daughter nuclei correctly activate the lat52-gus/nia marker and show disperse chromatin condensation.
Incomplete internal cell walls are often seen connected to the pollen grain wall. gem1 mutation has been tracked down to a microtubule associated protein with strong homology to the human chTOG and Xenopus XMAP215 family of microtubule associated proteins. GEM1 protein is involved in microtubule assembly and is associated with interphase, spindle and phragmoplast microtubule arrays (Park et al., 1998; Park and Twell, 2001, Twell et al., 2002). The two-in-one pollen (tio) mutant previously termed solo pollen mutant isolated from the morphological screen exhibits 50% of mature pollen grains containing a single nucleus, indicating failure to undergo cytokinesis at PMI. These mutations affect the ability of the microspore to divide. GUS positive staining of mutant tio pollen grains carrying the lat52-gus/nia gene show that the VC-specific promoter is activated, confirming that the VC fate is the default condition of the microspore nucleus in the absence of division at PMI (Twell and Howden, 1998).

A distinct class of mutant that affects integrity and/or the positioning of the male germ unit (MGU), male germ unit malformed (mud) and germ unit malformed (gum) pollen mutants, was also isolated from the morphological screen. In gum mutants, the vegetative nucleus is positioned adjacent to the pollen grain wall, separate from the two sperm cells, whereas in the mud mutants the intact MGU is displaced to the pollen grain wall. Genetic analysis revealed that the gum and mud act gametophytically and cytological analysis revealed that that correct MGU assembly is required for efficient transmission of the male gametes through the pollen (Lalanne and Twell, 2002).

A striking class of division mutant that emerged from the screen is the duo pollen (duo) mutants that produce bicellular pollen at anthesis instead of the stereotypical tricellular pollen grains. Mutant generative cells in duo mutants fail to undergo PMII. To date little is known about the division of the male germ line, the study of mutants exhibiting impaired PMII or failure to complete PMII will provide access to understand mechanisms underlying the controls of GC division.
1.19 Aims of this project

This work was initiated after the isolation of *duo pollen* mutants from the morphological screen described in section 1.18. The major objective of the project was to gain insight into the mechanisms controlling generative cell division in *Arabidopsis* and to isolate a gene required for generative cell division.

In the first part of the project, the specific aims of the experiments were to characterise six *duo pollen* mutants genetically. This involved the genetic transmission through the male and the female gametes, determine the gametophytic nature of the mutations and to assign a map position to each *duo* mutants (Chapter 3).

In the second part of the study, the specific aims of the experiments were to characterise three *duo* mutants, *duo1*, *duo2* and *duo3*, phenotypically. The precise point of development arrest was established and emphasis was laid on the generative cell division. A technique was developed to measure the DNA content in the generative nucleus and the sperm nucleus of the mutants as well as the wild type. Ultrastructure of the *duo* mutants was analysed by transmission electron microscopy. Cytological analysis such as vital staining, vegetative cell fate and pollen tube growth *in vitro* were monitored (Chapter 4).

In the final part of the project, emphasis was laid to isolate one of the *duo* genes. To analyse the molecular basis of the requirement of *DUO3* for generative cell division, *DUO3* gene was cloned using a map-based approach. The candidate gene was amplified and sequenced from genomic DNA of heterozygous *duo3* as well as from wild type. RT-PCR and bioinformatics searches were carried to find out about expression and the nature of the gene (Chapter 5).

Using the strategies described above, evidence is provided for gametophytic and genetic controls at PMII in *Arabidopsis*. This study has provided new insights into understanding the developmental programs associated with the S phase of the cell cycle.

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in the generative and sperm cell. Isolation of the *DUO3* gene revealed a unique gene in *Arabidopsis* that is required for progression into generative cell division.
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2.1 Chemicals used

Chemicals were purchased from the following company: Agar scientific, Sigma chemical company Ltd, Fisher Scientific, Qiagen, AB-gene. Molecular Biology reagents and enzymes were obtained from Gibco BRL, Bioline, New England Biolabs (NEB).

2.2 Plant materials

2.2.1 Growth conditions

*Arabidopsis thaliana* ecotype Nossen and Columbia were grown in 3:1:1 compost: vermiculite: sand mix under standard greenhouse conditions (16 hours light, 22°C). Seeds were surface sterilised with 70% (v/v) ethanol for 5 minutes and 10% (v/v) chloros bleach for 15 minutes, washed three times with distilled water and dried on filter paper for 30 minutes. Sterilised seeds were then plated on MS-0 media with or without selection.

2.2.2 Antibiotics used for plant selection

<table>
<thead>
<tr>
<th>Antibiotic name</th>
<th>working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefotaxime (Melford Lab, UK)</td>
<td>200 µg/ml</td>
</tr>
<tr>
<td>Gentamycin (Melford Lab, UK)</td>
<td>110 µg/ml</td>
</tr>
<tr>
<td>Kanamycin (Melford Lab, UK)</td>
<td>50 µg/ml</td>
</tr>
</tbody>
</table>
2.2.3 Storage of plant materials

For long-term storage, leaf tissues for DNA and RNA extraction were frozen in liquid nitrogen and kept in −80°C freezer. Buds were fixed in 3:1 95% (v/v) ethanol: acetic acid for 24 hours and stored in 75% (v/v) ethanol at 4°C.

2.3 Bacterial culture and storage

2.3.1 Bacterial strains

*Escherichia coli* (*E. coli*) XL1-Blue  
*Agrobacterium tumefaciens* (*A. tumefaciens*) GV3101

2.3.2 Growth conditions

*E. coli* and *A. tumefaciens* cultures were grown at 37°C and 28°C respectively either on plates in an incubator or in liquid medium on an orbital shaker at 200 rpm.

2.3.3 Bacterial media

Luria Bertani medium (LB): Bacto-tryptone 1% (w/v), bacto-yeast extract 0.5% (w/v), NaCl 1% (w/v). pH was adjusted to 7.0 with 5N NaOH and autoclaved for 15 minutes at 120°C. Solidified Luria Bertani medium (LB agar) was prepared as above and 1.5% (w/v) bacto-agar was added before autoclaving.

2.3.4 Antibiotics for bacterial selection

<table>
<thead>
<tr>
<th>Antibiotic name</th>
<th>working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (XL1Blue/pPZP221)</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td><em>A. tumefaciens</em> (GV3101/pPZP221)</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>-</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>100 µg/ml</td>
</tr>
<tr>
<td>Tetracycllin</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>15 µg/ml</td>
</tr>
</tbody>
</table>
2.3.5 Long-term storage of bacterial strains

A 0.5 ml aliquot of an overnight grown *E. coli* or *A. tumefaciens* culture was transferred to a cryogenic storage tube and was mixed with 0.5 ml filter sterilised 50% (v/v) glycerol. The cells were frozen in liquid nitrogen and stored at −80°C. Single colonies of each strain was recovered by streaking a small portion of the frozen culture on solidified media containing selection antibiotic and grown as described in section 2.3.2.

2.4 Isolation nucleic acids

2.4.1 Isolation of genomic DNA

About 2-3 leaf tissues were collected in a 1.5 ml microcentrifuge tube and were frozen in liquid nitrogen. About 200-300 glass beads (Sigma, UK) of size 600 μm were added to the leaf tissues and ground for 10 seconds in a silament amalgam mixer (Ivoclar Vivadent, UK). 250 μl of 3% (w/v) CTAB extraction buffer (1.4 M NaCl, 3% (w/v) CTAB, 20 mM EDTA, 100 mM Tris-Hcl pH8) was added and incubated for 15 minutes at room temperature after vortexing briefly. An equal volume of chloroform: IAA (24:1)(250 μl) was added and centrifuged for 10 minutes at 14000 g. The upper phase was transferred to a fresh microfuge tube, precipitated with 0.7 volume of isopropanol and centrifuged for 5 minutes at 14000 g. The supernatant was discarded, 500 μl of 70% (v/v) ethanol was added to the DNA pellet and centrifuged for 5 minutes at 14000 g. The supernatant was discarded and the pellet was vacuum dried for 5 minutes. The dried pellet was dissolved in 100 μl of DW.

2.4.2 Isolation of total RNA

TRIZOL reagent (GIBCO BRL) was used to isolate total RNA from mature leaves, stem, roots, flowers, microspores and pollen. The tissues were ground to a fine powder in liquid nitrogen and homogenise in 1 ml of TRIZOL Reagent per 50-100 mg of tissue. The tissues were vortexed briefly and the homogenised samples were incubated for 5 minutes at 30°C. 0.2 ml of chloroform per 1 ml of TRIZOL reagent was added,
vortexed for 15 seconds and incubated at 30°C for 2 to 3 minutes. The samples were centrifuged at 12000 g for 15 minutes at 2°C, following the centrifugation total RNA remained exclusively in the aqueous phase. After transferring the aqueous phase to a fresh tube, 0.5 ml of isopropyl alcohol was added per 1 ml TRIZOL Reagent used for the initial homogenisation, mixed and incubated at 30°C for 10 minutes. The samples were centrifuged at 12000 g for 10 minutes at 2°C, the supernatant was removed and the RNA pellet was washed with 1 ml of 75% (v/v) ethanol per 1 ml of TRIZOL Reagent. The samples were vortexed briefly and centrifuged at 7,500 g for 5 minutes at 2°C. The supernatant was discarded and the RNA pellet was vacuum dried for 5 minutes. The RNA pellet was dissolved in RNase-free water (DEPC treated water) by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55°C. Total RNA isolated was quantified at 280 nm using a Philips (PU 8740 UV/VIS) scanning spectrophotometer.

2.4.3 Small-scale isolation of plasmid DNA

According to Sambrook (1989) a single colony was picked and grown overnight in 3 ml LB medium. A 1.5 ml aliquot of the culture was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 14000 g for 5 minutes. The pelleted cells were resuspended in 100 µl of solution I (glucose 50 mM, Tris-HCl (pH 8.0) 25 mM and EDTA (pH 8.0) 10 mM). After adding 200 µl solution II (NaOH 0.2 N, SDS 1% (w/v)), the tube was gently inverted 5 times to lyse the bacterial cell and incubated at room temperature for 10 minutes. 150 µl of solution III (potassium acetate 5 M, glacial acetic acid 11.5% (v/v)) was added to the lysate, mixed, incubated on ice for 30 minutes and centrifuged at 14000 g for 10 minutes at 4°C. The supernatant was transferred to a fresh tube and an equal volume of 24:1 phenol/chloroform: isoamyl alcohol (100ml phenol, 100 ml chloroform and 4.16 ml isoamyl alcohol) was added, vortexed and centrifuged at 14000 g for 5 minutes. The top aqueous layer was transferred to a clean tube and 2.5 volumes of absolute ethanol was added and centrifuged at 14000 g for 5 minutes. The pellet was washed with 100 µl of 70% (v/v) ethanol and centrifuged at 14000 g for 1 minute. The pellet was vacuum dried for 5 minutes and resuspended in 30 µl of DW.
2.5 Amplification of DNA by polymerase chain reaction (PCR)

2.5.1 Primer design

Primers were designed to generate SSLP markers to amplify genomic DNA of approximately 1 kb in length and to amplify genomic sequence of genes that were used for cloning. The primers were designed using the programme primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3T).

The conditions used to design primers for SSLP markers were as follows: the length of the primers ranged between 22-26 bp with the melting temperature between 60°C to 67°C. GC content was maintained between 40-50% within the primers and at least 2 GC clamps were included in the 3' primer end. Product size for SSLP markers usually ranged between 150-200 bp. The primers used to amplify 1 kb genomic DNA were designed as for the SSLP markers but the product size was approximately 900-1200 bp.

Primers used to amplify 3 - 7 kb genomic DNA (genes) were designed as above however the length of the primers were increased to 35 bp and the melting temperature ranged between 65-70°C. GC content was maintained between 40-50% within the primers and at least 2 GC clamps was included in the 3’ primer end.

2.5.2 Oligonucleotide preparation

Dry oligonucleotides obtained from Sigma were resuspended in appropriate volumes of DW as recommended by the supplier. The primer stock (100 µm) was prepared and stored at −4°C.

2.5.3 PCR reactions

To amplify 150-1000 bp genomic fragments, the PCR reaction was set up in a 20 µl reaction mixture. The reaction mixture was kept on ice, mixed by vortexing and spun briefly. Taq-polymerase, dNTP, buffer and MgCl₂ were obtained from Bioline.
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**PCR reaction mixture**

- DNA template (genomic DNA) \(1.0 \mu l\)
- 20 µM primers forward \(0.5 \mu l\)
- 20 µM primers reverse \(0.5 \mu l\)
- 2 mM dNTPs \(2.0 \mu l\)
- 10X buffer \(2.0 \mu l\)
- 50 mM MgCl\(_2\) \(0.8 \mu l\)
- 5.0 U Taq polymerase \(0.2 \mu l\)
- DW \(13 \mu l\)

To amplify 3-7 kb fragment, Ex-Taq polymerase (Takara, Japan) was used and the reaction mixture was set up in 25 µl. The reaction mixture was kept on ice and all the components of the PCR reaction were kept at the bottom of the amplification tube.

**PCR reaction using Extaq polymerase**

- DNA template (genomic DNA) \(1.0 \mu l\)
- 20 µM primers forward \(1.0 \mu l\)
- 20 µM primers reverse \(1.0 \mu l\)
- 2.5 mM dNTP mixture (20 mM Mg\(^{2+}\)) \(2.0 \mu l\)
- 10X buffer \(2.5 \mu l\)
- 1.0 U Taq polymerase \(1.0 \mu l\)
- DW \(16.5 \mu l\)

2.5.4 DNA amplification

The PCR program used to amplify DNA fragments varied according to the size of the product and the melting temperature of the primers. The DNA template was initially denatured at 94°C for 2 minutes. Annealing temperature depended on the melting
temperature of the primers and extension time was set up at 72ºC for 30 seconds or 1 minute for every kb of target DNA fragment. The PCR program was set up as follows:

<table>
<thead>
<tr>
<th>Initial denaturation</th>
<th>96ºC</th>
<th>2 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thermal cycling</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>96ºC</td>
<td>15 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>55-65ºC</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Extension</td>
<td>72ºC</td>
<td>1 minute per kb</td>
</tr>
</tbody>
</table>

The thermal cycling step was repeated 39 times. The PCR product was analysed by agarose gel electrophoresis (section 2.7).

### 2.5.5 Colony PCR

A small portion of a single colony was picked with a sterile toothpick and inoculated into a 20 µl PCR reaction (section 2.5.3). Standard PCR amplification was performed as described in section 2.5.4.

### 2.6 Reverse Transcription PCR (RT-PCR)

RNA template used for RT-PCR was isolated with TRIZOL reagent (GIBCO BRL) as described in section 2.4.2. RT-PCR was performed using the AB-gene RT-PCR Kit (AB-gene, UK) to detect RNA transcripts in *Arabidopsis* stem, leaf, roots, flowers, microspore and pollen.

#### 2.6.1 RT-PCR reaction mixture

The RT-PCR reaction mixture was prepared in a 0.5 ml microfuge tube that was kept on ice, mixed and spun briefly. The presence of target template was detected by analysing the RT-PCR product using agarose gel electrophoresis (section 2.7)
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2x ReddyMix™ Master Mix 25 µl
RNA template (1 µg/ml) 1µl
Primers (10 µM) 1µl
Reverse transcriptase blend 1µl
DEPC treated DW made up to 50µl

2.7 Agarose gel electrophoresis for separation of DNA

50x TAE buffer: Tris base 242 g, glacial acetic acid 57.1 ml, EDTA 0.5 M (pH 8.0) 100 ml.
10x Gel loading buffer: Orange G 0.5% (w/v), glycerol 50% (v/v)

Based on a method from Sambrook et al., 1989, an appropriate amount of agarose, 4 g of agarose to make 4% gel or 2 g to make 2% gel, was melted in 100 ml of 1x TAE buffer. The molten gel was cooled to 60°C and ethidium bromide was added to make a final concentration of 0.5 µg/ml, mixed thoroughly and poured into a mould with a comb. The gel was allowed to set for 20 minutes at room temperature, the comb was carefully removed and the solidified gel was placed into an electrophoresis tank containing 1x TAE buffer. DNA samples were loaded into the wells after mixing it with 10X gel loading buffer to a final concentration of 1X. A voltage of 8V/cm was applied and the gel was run until the DNA fragments were completely separated. After electrophoresis, DNA was visualised using an UVP trans-illuminator and a thermal gel print was taken with a graphic printer (UP-895CE, Sony). The size and quantity of DNA fragments was determined by comparing a known fragment of standard DNA ladder (GIBCO BRL).

2.8 Purification of nucleic acids

2.8.1 Purification of DNA fragments from agarose gels

DNA fragments required for sequencing or cloning were purified from agarose gel using QIAquick DNA purification kits (Qiagen) according to manufacturers instructions. The DNA fragment was excised from the agarose gel with a clean, sharp
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scalpel. The gel slice was weighed and 300 µl of the buffer QG was added per 100 mg of the gel. The sample was then incubated at 50°C for 10 minutes and mixed by vortexing the tube every 2-3 minutes during the incubation. After the gel had dissolved completely, 100 µl of isopropanol was added per 100 mg of agarose gel slice. QIAquick column was placed in a 2 ml collection tube and the sample was applied to the QIAquick column to bind the DNA. After centrifugation for 1 minute at 13,000g, the flow through was discarded and the QIAquick column was placed back in the same collection tube. A 0.5 ml aliquot of buffer QG was added to the samples to remove all traces of agarose. The flow through was discarded and the QIAquick column was placed back in the same collection tube. A 0.75 ml aliquot of buffer PE was added to QIAquick column and centrifuged for 1 minute at 13,000g. After discarding the flow through the QIAquick column was centrifuged for an additional 1 minute at 13,000g. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube and 50 µl of buffer EB was added to the centre of the QIAquick membrane to elute the DNA. The column was allowed to stand for 1 minute and then centrifuged at 13,000g for 1 minute.

2.8.2 Purification of DNA

To remove the proteins from the DNA solutions following enzymatic manipulations of DNA, an equal volume of phenol/chloroform: isoamyl alcohol (24:1) was added to the reaction mixture, vortexed and centrifuged at 14000 g for 5 minutes. The aqueous phase was transferred to a fresh tube. The DNA was precipitated by adding two volumes of 95% (v/v) ethanol and sodium acetate (3 M, pH 5.2) to a final concentration of 0.3 M, mixed by brief vortexing and centrifuged at 14000 g for 10 minutes. The supernatant was discarded and the pellet was washed with 1 ml of 70% (v/v) ethanol and centrifuged at 14000 g for 1 minute. The supernatant was discarded, the pellet was vacuum dried for 5 minutes and resuspended in DW.

2.8.3 Sequencing of PCR product

The PCR products were supplied to Protein and Nuclei Acid Chemistry Laboratory (PNACL, Leicester University) for sequencing. The PCR product was purified as
described in section 2.8.1. To sequence one kb DNA fragment about 50-100 ng (8 µl) of PCR product in a 0.5 ml microcentrifuge tube was sent to PNACL. Either the forward or the reverse primers was supplied at a concentration of 1.0 p mol/µl. PNACL carried out the sequencing reactions, purified them using DyeEX columns and analysed the products using an ABI 377 automated sequencer. The sequencing information obtained was analysed using *gene jockey* (sequence processor, UK) and the chromatogram was viewed using the program *Sequence Editor™ version 1.03* (Applied Biosystems Inc)

### 2.8.4 Quantification of nucleic acids

DNA was quantified using size ladder (λ Hind III) (GIBCO BRL). Concentration of RNA was determined by measuring the UV absorbance of RNA samples at wavelengths of 280 nm using a Philips (PU 8740 UV/VIS) scanning spectrophotometer. The concentration of nucleic acid in the sample was calculated on the basis that an OD of 1 corresponds 40 µg/ml of single stranded RNA.

### 2.9 Enzymatic manipulation of DNA

#### 2.9.1 Digestion of DNA with restriction endonucleases

The amount of enzymes used for digests was less than 0.1 volume of the final reaction mixture to avoid inhibition of the enzyme activity by glycerol. The DNA digests were set up using 10U of restriction enzyme in a total volume of 30 µl for 1hr. The restriction enzyme buffer and the incubation temperature were set up according to the manufacturers instructions. In cases where two enzymes were used to digest a DNA sample and the reaction conditions were not favourable for simultaneous digests, the DNA was purified (section 2.8.2) after the first digest and the second digest was set up with the favourable buffer and incubation temperature.

#### 2.9.2 Dephosphorylation of linearised plasmid DNA
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To prevent self-ligation of the linearised plasmid DNA, the 5’-phosphates were removed using calf intestinal alkaline phosphatase (CIP). Following digestion of the plasmid DNA (1-1.5 μg) with the desired restriction endonuclease, 0.5 unit of CIP, 10 μl of 10X CIP dephosphorylation buffer and 40 μl of DW were added to make a final volume of 100 μl. The reaction was incubated at 37°C for 30 minutes. Purification of the dephosphorylated DNA was carried out by phenol/chloroform extraction and ethanol precipitation as described in section 2.8.2.

2.9.3 Ligation of DNA fragments

Plasmid vector and insert for a recombinant plasmid were ligated in a 10 μl ligation reaction. The ligation reaction consisted of 2 units 0.5 μl of T4 ligase (GIBCO BRL), 2 μl of 5X T4 ligase buffer (50 mM Tris-HCl, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 25 μg/ml BSA) with the concentration of termini of the insert approximately 3 fold higher than the plasmid vector. Approximately 60 ng of the vector DNA was used in ligations. The ligations were incubated overnight at RT and all the ligations were used for bacterial transformation as described in sections 2.15.

2.10 Cytological analysis

2.10.1 Staining pollen nuclei with 4’,6-diamidino-2 phenylindole (DAPI)

Pollen nuclei were visualised after staining with 4’,6-diamidino-2 phenylindole (DAPI) as described by Park et al., 1998. Mature pollen were collected by placing 3-4 fully open flowers in a microfuge tube containing 300 μl of 10 μg/ml DAPI staining solution (0.1 M sodium phosphate, pH 7; 1 mM EDTA, 0.1% (v/v) Triton X-100, 0.4 mg/ml DAPI; high grade, Sigma). Pollen grains were released into the DAPI solution by brief vortexing and centrifuged briefly for 2-3 seconds in a picofuge (Stratagene, UK) to form a pollen pellet. 5 μl of the pollen pellet was transferred to a microscope slide and viewed under epifluorescence microscope (Nikon Optiphot). For screening pollen from mapping population mature pollen from a single open flower was released into a well of
microtiter plate containing 100 μl of DAPI solution and pollen nuclei were visualised under an inverted epifluorescence microscope (Zeiss Axiophot).

For the analysis of spores at earlier stages, buds were separated and sequentially arranged based on their position on the floral axis. Single anthers were dissected using a dissecting microscope (Zeiss, Stemi SV8) to release the pollen grains into 5 μl of DAPI solution. A coverslip was mounted, gently squashed to flatten the samples, sealed with nail varnish and visualised under epifluorescence microscope.

### DAPI staining

- DAPI (10μg/ml) 8μl
- GUS buffer (0.1 M NaPO₄ pH 7.0, 0.5 M EDTA, 10% (v/v) Triton) 10 ml

#### 2.10.2 Vital staining of pollen grains

Pollen grain viability was determined cytologically by visualising cellular esterase activity with 5mg/ml fluorescein diacetate (FDA) (Heslop-Harrison and Heslop-Harrison, 1970). Pollen grains from 3-4 open flowers were collected into a microfuge tube and resuspended in 100 μl of freshly made FDA stain. Pollen grain were visualised under epifluorescence microscope (Zeiss Optiphot).

### FDA stain

- 0.3 M Mannitol 990 μl
- 5 mg/ml FDA (in acetone) 10 μl

#### 2.10.3 In vitro pollen germination

Stamens from five to ten fully opened flowers were transferred into a well of a culture plate containing 150 μl pollen germination media (0.01% (w/v) Boric acid, 0.07% (w/v) Calcium Chloride Di-hydrate, 3% PEG and 20% (w/v) sucrose). The plate was sealed with Nescofilm and incubated at room temperature on a shaker (30 rpm) for 8-10 hours.
2.10.4 GUS staining

To visualise GUS staining, pollen was incubated overnight at 37°C in GUS buffer (0.1 M sodium phosphate (pH 7), 10 mM EDTA, 0.1% (v/v) Triton X-100, 5 mM potassium ferricyanide) containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc; Biosynth).

2.11 Ultrastructural analysis

Material was prepared for thin sectioning essentially according to Owen and Makaroff (1995) and Park and Twell (2001). Plants were grown as described in section 2.2.1 and materials (inflorescences) were collected in Eppendorf tubes containing 1 ml fixative solution (section 2.11.1). The pedicels of the inflorescences were kept for easy handling.

2.11.1 Fixation

Whole inflorescence clusters were fixed overnight at room temperature in 1 ml fixative solution.

**Fixative solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutaraldehyde</td>
<td>2.8%</td>
</tr>
<tr>
<td>HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (pH 7.2)</td>
<td>0.1M</td>
</tr>
<tr>
<td>Triton X-100 (v/v)</td>
<td>0.02%</td>
</tr>
</tbody>
</table>

After overnight fixation the bud clusters were rinsed twice in 0.1M HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (pH 7.2) buffer for 15 minutes each, and post-fixed in 1% (w/v) aqueous Osmium tetraoxide (OsO₄) overnight. Following post-fixation with OsO₄ the tissues were washed with DW three times for 10 minutes each wash. Osmium tetraoxide is a harmful substance, therefore post-fixation with OsO₄ was carried out in a fume hood.
2.11.2 Dehydration

The tissues were dehydrated in a series of acetone (10% increments). The bud clusters were carefully transferred in graded series of 1 ml acetone solutions in tubes by holding the pedicel with a pair of forceps and incubated at RT for at least an hour as described below.

- 10% acetone: 90% DW at least 1 hr
- 20% acetone: 80% DW at least 1 hr
- 30% acetone: 70% DW at least 1 hr
- 40% acetone: 60% DW at least 1 hr
- 50% acetone: 50% DW at least 1 hr
- 60% acetone: 40% DW at least 1 hr
- 70% acetone: 30% DW at least 1 hr
- 80% acetone: 20% DW at least 1 hr
- 90% acetone: 10% DW at least 1 hr
- 100% acetone three times at least 30 minutes each time

After dehydration individual buds were dissected from the bud clusters and each bud was kept in an embedding capsule.

2.11.3 Infiltration and embedding

Buds were embedded in Spurr’s resin (Agar, scientific). Spurr’s resin is available as a kit and was prepared by mixing 10.0g VCD (ERL4206), 4.0g diglycidyl ether of polypropylene glycol (DER736), 26.0 g Nonenyl succinic anhydride (NSA) by continuous stirring for 10 minutes and 0.4 g dimethylamino ethanol (DMAE) was added and stirred for further 5 minutes. The samples were infiltrated with a graded series of 200 µl Spurr’s resin and acetone.

Infiltration of Spurr’s resin:

- 20% resin: 80% acetone 3 hrs
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<table>
<thead>
<tr>
<th>Resin Percentage</th>
<th>Acetone Percentage</th>
<th>Incubation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>40%</td>
<td>60%</td>
<td>3 hrs</td>
</tr>
<tr>
<td>60%</td>
<td>40%</td>
<td>3 hrs</td>
</tr>
<tr>
<td>80%</td>
<td>20%</td>
<td>3 hrs</td>
</tr>
<tr>
<td>100%</td>
<td></td>
<td>three times 3 hrs each time</td>
</tr>
</tbody>
</table>

Following infiltration with Spurr’s resin, the samples were transferred to a fresh embedding capsule using disposable pipette and refilled with 200 μl fresh Spurr’s resin. The samples were arranged in the centre on the bottom of the tube and the resin block was incubated at 60°C for 16 hrs.

2.11.4 Sectioning

One μm thick sections were cut on an ultramicrotome (OMU4, Reichert Jung, Leica, Milton Keynes, UK) and were stained with 0.5% (w/v) toluidine blue O in 2% (w/v) sodium borate for examination. Ultrathin sections were cut with a glass knife on an ultramicrotome and picked up on 300-mesh grids. Sections were stained with uranyl acetate (Agar, scientific, UK) and lead citrate (Fisher, scientific, UK) and were viewed with a transmission electron microscope (100CX, JEOL, Tokyo). Photographs were taken on film (SCIENTIA, 23D56 P3 AH, AGFA, Gevaert, Belgium). Negatives were scanned and processed using Adobe Photoshop 4.0 (Adobe Systems, Mountain View, CA).

2.12 Generation of tetraploid plants

Tetraploid plants were generated based the modified protocol of Vizir and Mulligan, 1999. About 30-50 seeds were placed in a microfuge tube containing 0.1 M potassium buffer (pH 5.8) and kept in the cold room for 3 days. The cold treated seed were incubated with 0.2% (w/v) colchicine in 0.1 M potassium phosphate buffer (pH 5.8) for 26 hrs at room temperature. At the end of the incubation, colchicine solution was removed and the seeds were washed three times with potassium nitrate solution 100 mg/L. The seeds were transferred to soil and grown as described in section 2.2.1.
2.13 DNA content measurement

2.13.1 Bud developmental stage

The relationship between bud stages and pollen development was determined. Bud stages were established based on their arrangement on the floral axis essentially as Lalanne and Twell, 2002. The first open flower was termed as +1 stage (mature tricellular pollen), the first and the second unopened flower buds were called −1 and −2 stage (mature undehisced pollen). The third unopened bud (late tricellular pollen) was termed −3 stage. The fourth (mid tricellular) and fifth (early tricellular) unopened flower buds were termed as the −4 and −5 stage respectively. The sixth unopened bud (bicellular pollen) was called as -6 stage. The seventh and eighth unopened bud were termed mid bicellular (-7 stage) and early bicellular stage (-8 stage) respectively.

2.13.2 Staining of spores and pollen

Buds clusters from wild type and mutant plants were fixed in 3:1 95% (v/v) ethanol: acetic acid for about 24 hours and were used immediately or were stored in 75% (v/v) ethanol at 4°C for one month. Buds were separated and anthers were dissected to release pollen into DAPI staining solution (as described in section 2.10.1). A coverslip was mounted, gently squashed to flatten the samples, sealed with nail varnish and kept in dark for 30 minutes. The samples were then visualised under an epifluorescence microscope.

2.13.3 Capturing images

The images were captured using a 3-CCD colour video camera (JVC, KY-F55B), linked to a Neotech IGPCI capture card and Image Grabber PCI 1.1 software on an Apple Macintosh computer. The settings to capture the images were kept standard. To capture DAPI pictures the microscope UV filter was on and the microscope light was switched off. On JVC controls panel the shutter was kept on slow and gain was set up to 0 dB. The FWA white balance was selected and six to ten frames were used. The picture was previewed on normal and then captured. To capture light images the UV filter was
closed and the microscope light was switched on. The diaphragm was fully opened. On the JVC control panel the shutter was selected on slow with 0 dB. The white balance was set on manual and six to ten frames were used.

2.13.4 Relative DNA measurements

Relative DNA contents of the generative and sperm cell nuclei were measured based on DAPI fluorescence emitted. The microscope was set up and images were captured as described in section 2.13.3. Images were analysed for fluorescence values using the Open lab3.1 software (Improvision, UK). For all measurements the relative fluorescence values were recorded using a fixed area. A region of interest was selected including a single nucleus and the reading was recorded. The net values for the nuclei were obtained by recording initial reading of the nucleus and subtracting a corresponding background reading from the cortical cytoplasm. In order to standardise the relative fluorescence per C value, the DNA content of the sperm was measured at telophase that by definition possesses a 1C DNA content. This data was used to calibrate the relative fluorescence per C unit.

2.14 Statistical Analysis

To determine whether the mean relative fluorescence of sperm nuclei and the generative nuclei were statistically significant at successive ontogenetic stage, one-tailed \textit{t-test} (Microsoft Excel software) assuming unequal variances was applied. The Chi-square (\(\chi^2\)) test was used to compare observed frequencies to expected frequencies if null hypothesis is true. The closer the observed frequencies are to the expected frequencies, the more likely the \(H_0\) is true. It is measured by squared difference relative to expected frequency. The test statistic is the sum of relative squared differences.

To test whether the proportion of wild type to mutant is the same (segregating 1:1), the following hypotheses were set up:
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\[ H_0: p_w - p_m = 0 \quad \text{where } i = \text{mutant plants} \]
\[ H_1: p_w - p_m = 0 \]

where \( p_w \) and \( p_m \) are proportions of wild type and mutant respectively.

The Chi-square test is:

\[
\text{chi} - \text{squared} = \frac{\sum (f_o - f_e)^2}{f_e} \text{ with } (r - 1)(c - 1) \text{ degrees of freedom where } f_o \text{ and } f_e
\]

are the observed and expected respectively. \( r \) is the number of rows and \( c \) the number of columns.

2.15 Bacterial transformation

2.15.1 Preparation of competent E. coli

Competent E. coli cells were prepared as described by Hanahan (1983). Single XL1-Blue colony was grown in 25 ml of LB (LB as in section 2.3.4, 18% (w/v) glucose 1 ml/100 ml and autoclaved 15 minutes at 120°C) containing tetracycline (10 mg/ml) at 30°C, 200 rpm. A 1 ml aliquot of the overnight culture was inoculated into 100 ml of pre-warmed LB along with tetracycline and allowed to grow at 30°C until OD_{600} reaches 0.2. 1 M \( \text{MgCl}_2 \) was added to a final concentration of 20 mM (2 ml of 1 M \( \text{MgCl}_2 \)) and the cells were grown until OD_{600} reaches 0.45-0.55. The bacterial cells were transferred to a sterilised 50 ml tube and incubated on ice for 30 minutes and centrifuged at 4°C, 5000 g for 5 minutes. The bacterial pellet was resuspended in 50 ml of prechilled \( \text{Ca}^{2+}\text{Mn}^{2+} \) solution (Sodium acetate 40 mM, \( \text{CaCl}_2 \) 100 mM, \( \text{MnCl}_2 \) 70 mM, pH adjusted to 5.5 with 1 M HCl and filter sterilised) and incubated on ice for 45 minutes. The bacterial cells were centrifuged at 5,000 rpm for 5 minutes, the supernatant was discarded and pellet was gently resuspended in 5 ml of \( \text{Ca}^{2+}\text{Mn}^{2+} \) solution containing 15% (v/v) glycerol. A 200 \( \mu \)l aliquot of the cells was dispensed into 1.5 ml tubes, frozen in liquid nitrogen and stored at −80°C.

2.15.2 Preparation of competent A. tumefaciens
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An *Agrobacterium* strain (GV3101) containing an appropriate helper T1 plasmid was grown in 5 ml of LB medium along with selective antibiotic overnight at 28°C on a shaker (200 rpm). A 2 ml aliquot of the overnight culture was added to 50 ml of LB medium in a 250 ml flask. The culture was grown at 28°C with vigorous shaking (250 rpm) until the OD$_{600}$ reaches 0.5-1.0. The cell suspension was chilled on ice and centrifuged at 3000 g for 10 minutes at 4°C. The supernatant was discarded and the cells were resuspended in 1 ml of 20 mm ice-cold CaCl$_2$ solution. A 100 μl aliquot of the cells was quickly dispensed into pre-chilled microfuge tubes, flash frozen in liquid nitrogen and stored at -80°C.

2.15.3 Transformation of *E. coli*

An aliquot of competent *E. coli* (section 2.15.1) was thawed on ice, plasmid DNA resulting from ligation reaction (section 2.9.3) was added, gently mixed by flicking the tube and incubated on ice for 30 min. The cells were heat shocked at 37°C for 5 min, transferred to 1 ml of LB (without antibiotic selection) in a falcon tube and grown on a shaker at 200 rpm for 1 h at 37°C. The resulting cells were spread on LB agar plates containing antibiotic selection, allowed to dry and incubated overnight at 37°C. The resulting transformants were identified by restriction digests (section 2.9.1) or by colony PCR (section 2.5.5).

2.15.4 Transformation of *A. tumefaciens*

A 100 μl aliquot of competent *A. tumefaciens* (section 2.15.2) was thawed on ice, 1 μg of plasmid DNA in a volume of 10 μl of DW was added and mixed by gentle flicking. The cells were quickly frozen in liquid nitrogen and thawed by incubating the tube in a 37°C water bath for 5 minutes. The cells were inoculated into 1 ml of LB (without antibiotic selection) in a sterile falcon tube and grown on a shaker at 200 rpm for 3-5 hrs at 28°C. After the incubation period, the cells were transferred in a microfuge tube and centrifuged for 30 seconds at 7000 g. The supernatant was discarded and the cells were resuspended in 100 μl of LB medium. The cells were spread on a LB agar plate
containing appropriate antibiotic selection, allowed to dry and incubated at 28°C for 2 days.

2.15.5 Transformation of *Arabidopsis*

*Arabidopsis* plants were transformed by floral dipping and were grown under long day light regime. The primary bolt was clipped when the plants were approximately 2-5 cm tall. After 1 week, the siliques and fully open flowers were removed and the plants were ready for transformation. A single transformed *Agrobacterium* colony was selected and inoculated in 5 ml of LB containing appropriate antibiotic, the cells were allowed to grow overnight at 28°C. A 1ml aliquot of the overnight culture was diluted in 400 ml of fresh LB media and the cells were grown for another 24 hrs in a shaker at 200 rpm at 28°C. The cells were centrifuged at 5000 rpm for 10 minutes and the cells were resuspended in standard infiltration medium.

**Infiltration medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Half strength of MS salts (Sigma, UK)</td>
<td>2.165 g/L</td>
</tr>
<tr>
<td>Full strength of Gamborg B5 vitamins (Duchefa, Netherlands)</td>
<td>3.16 g/L</td>
</tr>
<tr>
<td>2-[N-Morpholino]ethanesulfonic acid (MES) (Sigma, UK)</td>
<td>0.5 g/L</td>
</tr>
<tr>
<td>Sucrose (Sigma, UK)</td>
<td>50 g/L</td>
</tr>
<tr>
<td>Benzyaminopurine (1mg/ml)(Sigma, UK)</td>
<td>10 μL/L</td>
</tr>
</tbody>
</table>

Just before dipping, 300 μl of silwet L-77 were added per litre of culture. The above ground part of plant was dipped in *Agrobacterium* solution for 2 minutes with gentle shaking. A plastic dome was kept on the dipped plants to keep the humidity high for one day. The plants were watered after two days.
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3.1 Introduction

Mature *Arabidopsis* pollen is a tricellular structure bearing a large vegetative cell and two smaller sperm cells. This tricellular architecture is achieved following two successive mitotic divisions. The first mitotic division (pollen mitosis I) is a highly asymmetric division and culminates in the production of two unequal daughter cells, the vegetative and the generative cell. The generative cell represents the male germ line and undergoes the second mitotic division (pollen mitosis II) to form twin sperm cells. Mutations affecting one of the mitotic divisions during pollen development would disrupt the stereotypical organisation of mature pollen grains. In order to identify such mutants a large-scale morphological screen was undertaken (Park et al., 1998). Approximately 10,000 M2 individuals from an EMS-derived *Arabidopsis* population (ecotype Nossen) were screened by examining DAPI-stained pollen under epifluorescence microscopy (section 1.18). This screening strategy led to the identification of a unique class of mutant termed *duo pollen* (*duo*) mutants. Six individuals termed *duo1, duo2, duo3, duo4, duo5* and *duo6* arising in different M1 parental groups were identified. At maturity *duo* mutants shed bicellular pollen grains, containing a vegetative nucleus and an undivided generative cell. The *duo* mutants were classified into two main groups based on the shape of the generative nucleus. The first group comprised of *duo1, duo2* and *duo3* that borne round generative nuclei at anthesis. The second class included *duo4, duo5* and *duo6* whereby the generative nuclei are frequently elongated (Figure 3.1). The *duo* pollen phenotype is indicative of pollen that has failed to enter or complete division at pollen mitosis II. All the six *duo* mutants were backcrossed as female to wild type (Nossen) plants to remove any background mutations. This chapter describes the genetic characterisation of all the six *duo* mutants. For each mutant selfing and reciprocal crosses were carried out. Tetrad analysis was performed for *duo3* and *duo6* to confirm the gametophytic nature of the mutations. A chromosomal location was assigned to each *duo* mutant.
3.2 duo pollen mutants were isolated as heterozygotes

All six duo mutants were backcrossed as females to wild type (Nossen) plants and the phenotype of the resulting progeny (BC₂ progeny) was screened. Fully opened flowers were collected from each BC₂ progeny and the pollen was released in DAPI solution. The DAPI-stained pollen grains were viewed under an epifluorescence microscope and nuclear morphology of the pollen grains was scored. Two types of progeny were scored for each mutant, wild type and duo plants. Mutant duo₁, duo₂, duo₃, duo₄, duo₅ and duo₆ plants from the backcrossed line (BC₂) were allowed to self fertilise and the proportion of wild type to mutant plants was scored.

The selfed progeny of duo₁ was screened for pollen phenotype and produced 101 wild type plants to 89 mutant plants. For duo₂ and duo₃ approximately 295 and 323 F₂ progeny were screened respectively that segregated approximately 50% mutant and 50% wild type plants. In case of duo₄, duo₅ and duo₆ the percentage of mutant plants was about 41% (n =155), 43% (n =149) and 41% (n =110) respectively. In all the six duo mutants the progeny in the selfed line segregated for wild type to mutant in the ratio of either 1:1 or 1.2:1 (Table 3.1). Chi-squared test ($\chi^2$) showed that these ratio do not differ significantly from 1:1 ratio, $P =0.005$ when $\chi^2 =3.84$. Expression of duo phenotype in the test cross progeny indicated that the duo mutations were originally isolated as heterozygotes and were predicted to act gametophytically or result from dominant sporophytic mutations.

3.3 Gametophytic nature of duo mutants

A fully penetrant male gametophytic mutation should exhibit a maximum of 50% mutant phenotype in a heterozygous plant (Park et al., 1998). In heterozygous duo₁, duo₂, duo₃ and duo₄ the percentage of aberrant pollen ranged between 47-49% (n >500) and the remainder of the population showed wild type phenotype, indicating that these mutants are expressed gametophytically and are fully penetrant. However, heterozygous duo₅ and duo₆ mutants exhibited only 31% (n =668) and 30% (n =723) of aberrant pollen respectively, showing reduced penetrance (Table 3.2). The proportion of
mutant pollen did not vary significantly in plants grown in different environments. Furthermore, no obvious sporophytic phenotypes were observed in the heterozygotes.

To confirm the gametophytic nature of \textit{duo} mutations tetrad analysis was performed using the \textit{quartet} \textit{l} (\textit{qrt}) mutants. Tetrad analysis was performed on \textit{duo}3 and \textit{duo}6 to confirm the gametophytic nature of the mutations. The \textit{qrt} \textit{l} mutant is a sporophytic recessive mutation that keeps all the products of a single meiosis together throughout pollen development (Preuss et al., 1994). In tetrad analysis if a mutation is sporophytically expressed but has low expressivity, the number of normal and affected pollen resulting from a single meiosis will vary. However, when heterozygous plant for a gametophytic mutation is introduced into \textit{qrt} (\textit{qrt}/\textit{qrt}) background, two members of the tetrad would show mutant phenotype. Heterozygous \textit{duo}3 and \textit{duo}6 plants (+/+; \textit{duo}/DUO) were crossed as female into \textit{qrt} (+/+; \textit{qrt}/\textit{qrt}) plants. Whereas 100% (n =168) of tetrads from homozygous \textit{qrt} \textit{l} plants appear wild type, in +/\textit{duo}3: \textit{qrt}/\textit{qrt} approximately 98% (n =104) of the tetrads contained two aberrant pollen and 2% (n =2) contained single aberrant pollen (Figure 3.2). In \textit{duo}6, 29% (n =55) of the tetrads all the members are wild type, 48% (n =91) of the tetrads contained two aberrant and two wild type pollen grains and 23% (n =44) of the tetrads had three wild type pollen grains and one aberrant pollen grain. The absence of tetrads with more than two aberrant pollen grains confirmed the gametophytic role of \textit{duo}3 and \textit{duo}6.

3.4 Genetic transmission

Genetic transmission of \textit{duo} through the male and the female gametes was determined by carrying out reciprocal test crosses in which heterozygous \textit{duo} mutants were crossed to wild type (Nossen) plants and the pollen phenotype of the progeny were scored. From a test cross progeny the transmission efficiency (TE) was calculated. TE of the mutant allele through the male or female gamete describes the fraction of mutant alleles that are successfully transmitted to the progeny (Howden et al., 1998). If the mutant allele is transmitted with 100% efficiency, test cross progeny should segregate 1:1 for wild type to mutant plants. This is based on the assumption that there are equal number of both alleles and random segregation of the alleles at meiosis. In \textit{duo}1, \textit{duo}2 and \textit{duo}3 there
Figure 3.1: Nuclear morphology of the *duo pollen* mutants.
(A) Wild type pollen grain with a diffusely stained vegetative nucleus and two strongly stained sperm nuclei (sperm nuclei are indicated by white arrowheads). (B), (C) and (D) represent *duo1*, *duo2* and *duo3* respectively with diffusely stained vegetative nucleus and a strongly stained, round generative nucleus (generative nucleus is indicated by white arrowhead).
(E), (F) and (G) show representative classes of *duo4*, *duo5* and *duo6* respectively, the vegetative nucleus is diffusely stained and the generative nucleus is elongated (generative nucleus indicated by white arrowhead).
Table 3.1: Number of wild type and mutant progeny from selfed heterozygous duo plants.

*The Chi-squared test ($\chi^2$) shows that these ratio do not differ significantly from 1:1 ratio (wild type to duo mutant plants), $\chi^2 = 3.84$ (P=0.005)
### Table 3.2: Frequency of pollen phenotypic classes in *duo1-duo6*.

The number of pollen in each phenotypic class is shown. Counts were made from heterozygous individuals. Data is derived from >500 pollen counted from four backcross progeny.
Figure 3.2: Tetrad analysis of duo3 and duo6
(A) In +/-;qrtl/qrtl all four members of the tetrad show a wild type phenotype
(B) In +/-;qrtl/qrtl two members of the tetrad show a mutant phenotype
(C) In +/-;qrtl/qrtl a maximum of two members of the tetrad show a mutant phenotype
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were no transmission through the male gamete. When heterozygous duo4, duo5 and duo6 were crossed as male, the transmission of duo relative to the wild type allele were 2.2%, 1.9% and 31% respectively indicating that these mutations have reduced transmission through the microgametophyte. However, in all six mutants transmission through the megagametophyte was normal (Table 3.3). These results demonstrate that the duo mutations specifically affect male gametophyte development, but do not compromise the functions of the female reproductive structures.

3.5 duo1, duo2 and duo3 mutations are recessive.

To find out whether the duo mutations are acting recessively or in a dominant manner tetraploid plants (4N) with the genotype DUO/duo' DUO/duo were generated that produce diploid pollen with the genotypes: DUO/DUO, DUO/duo and duo/duo. Tetraploid plants were constructed after treating duo1, duo2 and duo3 seeds with colchinine (section 2.12) and tetraploid sectors of the plants are generated (Vizir and Mulligan, 1999). Putative tetraploid plants were preselected visually at the seedling stage on the basis of enlarged width of cotyledons and putative tetraploid chimeric inflorescence sectors were selected on the basis of enlarged flower size grain. Tetraploid pollen grains were screened on the basis of enlarged pollen grains under the microscope. The wild type pollen grains segregate from the duo pollen mutant in duo1, duo2 and duo3 with a ratio of 3:1 in the tetraploid plants, indicating that the duo mutations are recessive (Table 3.4).

3.6 Chromosomal location of duo mutations

3.6.1 Mapping population

Mapping populations were generated by out crossing heterozygous duo plants as female (Nossen ecotype-No-0) to wild type (Columbia ecotype-Col-0). The F1 plants segregated into wild type and mutant plants in the ratio 1:1. F1 plants heterozygous for duo mutant were identified by screening DAPI-stained pollen and were allowed to self-fertilise. Self-fertilised plants produced both wild type and duo mutant plants (Figure
3.3). Wild type F2 plants were used as the mapping population. Approximately 50 wild type plants were used for detecting linkage.
### Table 3.3. Genetic transmission analysis of duo mutations

Reciprocal crosses between heterozygous mutants (+/duo) and the wild type (+/+ ) are depicted. The number of wild type and mutant progeny is shown along with the calculated transmission efficiency (TE, number of mutant/ number of wild type progeny x 100) through the female (TE\(^F\)) and male (TE\(^M\)) gametes.

* TE values that do not significantly differ from the expected value of 100%, \( \chi^2 = 10.83 \) (p =0.001).

\( \chi^2 = 10.83 \) (p =0.001).
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Tetraploid plants with the genotype DUO/duo segregated into wild type and duo pollen grains in the ratio 3:1. The Chi-squared ($\chi^2$) test shows that the ratio of wild type to duo pollen does not significantly differ from 3:1 ratio, $\chi^2 = 10.83$ (p =0.001).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild type (DUO/DUO)</th>
<th>duo (DUO/duo)</th>
<th>% duo</th>
</tr>
</thead>
<tbody>
<tr>
<td>DUO1/duo1</td>
<td>78</td>
<td>19</td>
<td>19.6</td>
</tr>
<tr>
<td>DUO2/duo2</td>
<td>144</td>
<td>31</td>
<td>17.8</td>
</tr>
<tr>
<td>DUO3/duo3</td>
<td>120</td>
<td>23</td>
<td>16.0</td>
</tr>
</tbody>
</table>

Table 3.4: Distribution of pollen phenotypic classes in tetraploid duo1, duo2 and duo3.
Figure 3.3: Schematic representation of the *duo* mapping population

Heterozygous *duo* (+/*duo*) plants were crossed as female to wild type Columbia plants. The F1 plants segregated into wild type and mutant plants in the ratio 1:1. Mutant (*duo*) F1 plants were allowed to self-fertilise. Wild type F2 plants were used as the mapping population. Col-0 = Columbia, No-0 = Nossen
3.6.2 Molecular markers used to establish linkage

A high density of genetic markers was required to establish linkage between markers and the duo mutations. Molecular markers that were used for mapping are simple sequence length polymorphism (SSLP) (Bell and Ecker, 1994) and cleaved amplified polymorphisms (CAPS) (Konieczny and Ausubel, 1993). The SSLP and CAPS markers were used because they are co-dominant therefore both chromosomes of a plant could be genotyped. Moreover the markers are PCR- based making it easy to use. A collection of SSLP and CAPS markers is available from The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/cgi-bin/maps/Schrom) database and initially these ready-made markers were selected to establish linkage. An extensive collection of polymorphisms is available between the ecotypes Columbia (Col-0) and Landsberg (Ler) but polymorphisms between Nossen (No-0) and Col-0 have not yet been established in the database. In order to find polymorphisms between the ecotypes Col-0 and No-0 new molecular markers were designed using the Col-0 sequence. TAIR exhibits all the simple-sequence repeats that are longer than 30 nucleotides in length, along with 200 bp flanking the repeated region. Primers were designed across the repeated regions using the primer design programme Primer 3 as described in Chapter 2 section 2.5.1 and the size difference between Col-0 and No-0 was determined.

3.6.3 Determining linkage

To identify markers that are genetically linked to the duo mutations, about 25 markers spanning the genome were exploited. The mapping population were genotyped using the markers (Figure 3.4). Linkage was determined based on recombination events. For example, if two loci were on different chromosomes they would segregate independently, but if they were linked to each other they would segregate together during meiosis and the recombination frequency would decrease. The duo mutations are in the Nossen background and wild type plant are homozygous for Col-0 alleles (C/C) at the duo locus. Among the wild type plants, unlinked markers would segregate independently from duo3 locus and the frequency of Col-0 (C) and No-0 (N) alleles would be approximately equal. The frequency of C alleles would be greater than the
number of N alleles for linked markers. The frequency of recombination events was estimated and these estimates served to assign a chromosomal location to the duo mutations. Percentage recombination frequency between duo and any marker was calculated by dividing the number of N alleles by the total number of alleles and multiplied by 100.

Chromosomal location of duo1 was established on chromosome 5 between the SSLP markers CTR1 and nga52 (Vizir and Twell, unpublished). duo2 showed 25% recombination frequency with the marker FIP2 on chromosome 3 and markers lying north and south of FIP2 were analysed. Based on the recombination frequency obtained from these flanking markers, it was established that duo2 lies south of FIP2 between the markers RPF24 (on BAC F24G16) and nga112 (on BAC T12C14) with recombination frequencies of 1.04% and 6.25% respectively. duo3 showed 15% recombination with the marker nga280 and duo3 locus was defined on the lower arm of chromosome 1, flanked by the SSLP markers F15H21 and F12A21. Recombination frequencies placed duo3 approximately 2.5 cM south of F15H21 and approximately 7.5 cM north of F12A21. duo4 showed 7.6% recombination with the marker nga139 on chromosome 4 and 2.1% recombination with the marker F16G20 which lies north of nga139 (Figure 3.4).

To identify markers that are genetically linked to duo6 a different approach was taken. Bulked segregant analysis is a faster way to identify linkage as it reduces the number of PCR reactions (Michaelmore et al., 1991). DNA was prepared from individual leaf samples and aliquot of the DNA was pooled. Two samples were prepared by pooling the DNA extracted from about 15 wild type plants. Both samples were examined with a collection of approximately 17 SSLP markers spaced evenly over the entire genome. One of the molecular markers F16G20 showed a clear bias toward the Columbia-specific band in the wild type pool (Figure 3.5). This indicates that the mutation maps to the lower arm of chromosome 4. Individual DNA samples were tested for linkage and it was confirmed that duo6 is linked to the marker F16G20 with a recombination frequency of 17.5%. Bulked segregant analysis does not allow the determination of the order of
Figure 3.4: Schematic representation of molecular markers and chromosomal locations of the DUO genes.

The horizontal bars indicate the unlinked markers that were used for mapping. The numbers in italics represent the recombination frequency and the coloured tick markers represent markers that showed linkage. The chromosomes (gray lines) were drawn using the chromosome map tool available from TAIR (http://www.arabidopsis.org)
Figure 3.5: Bulked segregant analysis of *duo6*.
Gel electrophoresis of PCR products for the SSLP markers, *nga280*, *nga1107* and *F16G20*. Lane 1 shows the homozygous Col-0 control sample (CC); Lane 2 shows the homozygous No-0 control sample (NN); Lane 3 represents the heterozygous control (CN). Lane 4 and 5 represent the pooled F2 samples. The mutation is linked to the marker *F16G20* as judged by the clear bias towards amplification of Col-0 band, shown by the white arrow. At the unlinked loci (with the markers *nga280* and *nga1107*) the ratio of Col-0 to No-0 is approximately the same as the heterozygous control.
closely linked loci on the chromosome. Therefore, individual DNA samples were tested for linkage with markers lying north and south of the marker \textit{F16G20}. The north marker, \textit{Ag}, showed 41.8\% recombination frequency and the south marker \textit{ngal139} revealed 9.52\% recombination frequency. Chromosomal location of \textit{duo6} was defined between the markers \textit{ngal139} with 9.52\% recombination frequency and the marker \textit{ngal107} with recombination frequency 30.2\%. The chromosomal location of \textit{duo5} was not determined and it did not mapped to any of flanking markers that were analysed.

3.6.4 Genetic maps of \textit{duo}

The genetic distance between the \textit{duo} mutations and linked markers were established. The genetic distance is defined as the average number of crossing over events during meiosis between two loci, involving a given chromatid (Koornneef and Stam, 1992). As the recombination frequency increases the map distance increases. Haldane (1919) and Kosambi (1944) established a mathematical relation between the recombination frequency and the map distance. To convert the estimated recombination frequency into map distances requires the conversion of the standard error. The standard error (S\textsubscript{r}) is an estimate of the recombination percentage.

The S\textsubscript{r} with different markers were calculated using the formula:

$$S_r = \sqrt{\frac{r(100 - r)}{n}}$$

where \textit{r} is the recombination percentage and \textit{n} represents the number of chromosomes.

The map distance was calculated using the formula:

$$D = -50 \ln(1 - \frac{r}{50})$$

Where \textit{D} is in centimorgans (cM) and \textit{r} is the recombination percentage

The standard error of the map distance was calculated based on the following formula:

$$S_D = \frac{50}{50 - r} S_r \text{ where } S_D \text{ is in cM}$$

Using the above formulas, genetic map positions of \textit{duo2}, \textit{duo3}, \textit{duo4} and \textit{duo6} genes were determined (Table 3.5).
Table 3.5: Genetic maps of \textit{duo}2, \textit{duo}3, \textit{duo}4 and \textit{duo}6

RF = recombination frequency and \( n \) = the number of chromosomes.

A) Genetic map of \textit{duo}2

<table>
<thead>
<tr>
<th>( duo2 )</th>
<th>( RPF24 ) RF=1.04, ( n=96 )</th>
<th>( nga112 ) RF=6.25, ( n=96 )</th>
<th>( RPF24-nga112 ) RF=7.29, ( n=192 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( S_r )</td>
<td>( D )</td>
<td>( S_D )</td>
<td>( S_r )</td>
</tr>
<tr>
<td>1.03</td>
<td>1.05</td>
<td>1.05</td>
<td>2.47</td>
</tr>
</tbody>
</table>

\[7.87 \pm 2.2\]

\[1.05 \pm 1.05\]

\[6.67 \pm 2.8\]

---

B) Genetic map of \textit{duo}3

<table>
<thead>
<tr>
<th>( duo3 )</th>
<th>( F15H21 ) RF=2.5, ( n=80 )</th>
<th>( F12A21 ) RF=7.5, ( n=80 )</th>
<th>( F15H21-F12A21 ) RF=10, ( n=160 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( S_r )</td>
<td>( D )</td>
<td>( S_D )</td>
<td>( S_r )</td>
</tr>
<tr>
<td>1.74</td>
<td>2.6</td>
<td>1.83</td>
<td>2.9</td>
</tr>
</tbody>
</table>

\[11.1 \pm 2.8\]

\[2.6 \pm 1.83\]

\[8.12 \pm 3.4\]
C) Genetic map of *duo4*

<table>
<thead>
<tr>
<th>duo4</th>
<th><em>F</em>16<em>G</em>20</th>
<th></th>
<th>nga1139</th>
<th></th>
<th><em>F</em>16<em>G</em>20-nga1139</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RF=2.17, n=46</td>
<td>S_r D S_D</td>
<td>RF=7.6, n=52</td>
<td>S_r D S_D</td>
<td>RF=9.77, n=98</td>
<td>S_r D S_D</td>
</tr>
<tr>
<td></td>
<td>2.14 2.21 2.23</td>
<td>3.67 8.24 4.3</td>
<td>2.98 10.87 3.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Chromosome IV

D) Genetic map of *duo6*

<table>
<thead>
<tr>
<th>duo5</th>
<th>nga1139</th>
<th></th>
<th>nga1107</th>
<th></th>
<th>nga1139-nga1107</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RF= 9.52, n=41</td>
<td>S_r D S_D</td>
<td>RF=30.2, n=96</td>
<td>S_r D S_D</td>
<td>RF=39.72, n=137</td>
<td>S_r D S_D</td>
</tr>
<tr>
<td></td>
<td>4.5 10.5 5.55</td>
<td>4.68 46.3</td>
<td>11.8 4.18 79</td>
<td>20.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Chromosome IV

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Chapter Three  Genetic analysis

3.7 Discussion

3.7.1 Isolation of the duo pollen mutants

Mutagenesis screens adopted to identify mutants affected in the gametophytic phase of the life cycle have proven to be very useful. Evidence for gametophytically acting genes affecting microgametogenesis has emerged from the genetic analysis of deficiencies in maize (Kindiger et al., 1991). To date a few male gametophytic mutants such as sidecar pollen, gemini pollen mutants, mad mutants, raring-to go, mud and gum have been isolated in Arabidopsis (Chen and McCormick, 1996; Grini et al., 1999; Johnson and McCormick, 2001; Lalanne and Twell, 2002). These gametophytic mutations provide further evidence of haploid expressed genes that specifically control gametophyte development. The duo pollen mutants represent a unique class of gametophytic mutants that is required during generative cell division.

3.7.2 duo pollen mutants are male specific gametophytic mutants

The duo pollen mutants represent a novel class of gametophytic mutants that specifically block generative cell formation. duo phenotype is expressed in the test cross progeny indicating that the duo mutations were originally isolated as heterozygotes and were predicted to act gametophytically or result from dominant sporophytic mutations. In each of the duo mutants the proportion of mutants in the F2 (self) progeny was approximately 50%, less than the expected 75%, suggesting reduced gametophytic transmission for each duo mutation. If a mutation inactivates a post-meiotically expressed gene that is essential for male or female gametophytic development, then the ratio of wild type to mutant plants in selfed progeny would be 1:1. Expression of 50% or less than 50% mutant pollen grains in heterozygous duo1, duo2, duo3, duo4, duo5 and duo6 along with tetrad analysis confirmed the gametophytic role of duo. Transmission via the male is impaired showing it is required specifically for the development of the male gametophyte. Genetic transmission through the female is normal in all the duo mutants indicating that the development or functions of the megagametophyte is not affected.
3.7.3 *duo1, duo2, duo3, duo4, duo5 and duo6 are independent mutations*

The *duo* mutants share very similar but distinct phenotype. When independently isolated mutants display an identical phenotype or very similar phenotype they may either result from mutations in the same gene (allelic) or from mutations in different genes (non-allelic). Allelism test can be performed to find out if two recessive mutants are representing different same gene or are different genes. When two allelic mutants are crossed, their F1 hybrid has a mutant phenotype. If they are non-allelic the F1 hybrid has a wild type phenotype, that is, the two mutants complement each other. However, determination of allelism for gametophytic mutants is laborious and requires the generation of tetraploid trans-heterozygotes that produce diploid gametophytes (Kamps et al., 1996; Grossniklaus et al., 1998). If the gametophytic mutations are transmitted to certain extent, diploid trans-heterozygotes mutants can be produced and an indication of how closely linked the loci are can be obtained. Allelism test could not be performed for the *duo* mutants due the gametophytic nature of the mutation and impaired transmission through the male gamete. The other alternative was to assign a map position for all the *duo* mutations. Mapping data revealed that the *duo1-duo6* represent different genes as they all mapped to different chromosomal locations. The map position of *duo5* was not determined and it was not to be allelic to the other *duo* genes. At least six different genetic loci control the unique division that leads to sperm cells formation in *Arabidopsis*. 
Chapter Four

Phenotypic analysis of duol, duo2 and duo3
Chapter Four

Phenotypic characterisation of \textit{duo1}, \textit{duo2} and \textit{duo3}

4.1 Introduction

Morphological screening of an EMS-mutagenised population yielded six \textit{duo} mutants that produced bicellular pollen grains at anthesis (described in section 1.18). Three \textit{duo} mutants, \textit{duo1}, \textit{duo2} and \textit{duo3} were selected for detailed phenotypic analysis because they share common characteristics. All three \textit{duo} mutants exhibit similar phenotype; the generative cells fail to enter or complete pollen mitosis II and the undivided round generative cells remain suspended in the vegetative cytoplasm. Moreover, genetic analysis revealed that \textit{duo1}, \textit{duo2} and \textit{duo3} are highly penetrant mutations. Heterozygous \textit{duo1}, \textit{duo2} and \textit{duo3} plants exhibit approximately 48\% mutant pollen phenotype at maturity (section 3.3).

The phenotypic characterisation and developmental analysis of \textit{duo1}, \textit{duo2} and \textit{duo3} are presented in this chapter. Nuclear morphology of the mutant pollen grains was analysed using epifluorescence microscopy and ultrastructural details were studied by transmission electron microscopy. Vegetative cell fate and viability of the mutants were assessed. Ontogeny of the pollen grains in the \textit{duo} mutants as well as in the wild type was followed to determine the precise point of development deviation. A technique was developed to measure the nuclear DNA content of the wild type generative and sperm cells during development. Given the efficacy of the technique nuclear DNA content of mutant generative cells were monitored.

4.2 \textit{duo} pollen mutants possess a single generative-like nucleus and a vegetative nucleus

To determine the external morphology and nuclear phenotype of the mutant pollen grains, mature pollen from heterozygous \textit{duo1}, \textit{duo2} and \textit{duo3} plants and wild type plants were stained with DAPI solution and were visualised under light and
epifluorescence microscopy. Pollen grains from all three \textit{duo} mutants were similar in size and appearance to wild type pollen under light microscopy (Figure 4.1A). DAPI staining combined with epifluorescence microscopy revealed that \textit{duol}, \textit{duo2} and \textit{duo3} exhibited two classes of pollen grains. Approximately 50\% (n=600) of the pollen grains from all three heterozygous \textit{duo} plants possessed only two nuclei and the remainder of the population exhibited the stereotypical tricellular pollen phenotype (Figure 4.1B). Mutant \textit{duo} pollen grains contained a large nucleus with diffuse DAPI staining, typical of the vegetative nucleus and a second smaller nucleus with more condensed chromatin similar to the generative nucleus.

Detailed analysis revealed that the nuclear morphology of the generative-like nuclei in \textit{duol} was significantly different from the generative nuclei in \textit{duo2} and \textit{duo3}. The generative-like nuclei in mutant \textit{duol} pollen were highly compact and irregular in structure. Moreover, distinct groups of condensed chromosomes with a mitotic morphology were commonly observed (Figure 4.2 D, E). In marked contrast in \textit{duo2} and \textit{duo3} the generative-like nuclei always appeared round, less compact than \textit{duol} but contained some localised regions of condensed chromatin (Figure 4.2 B, C). In all three mutants the vegetative nucleus and the generative-like nucleus usually occupied a central location and remained in close association with each other suggesting that the male germ unit (MGU) is formed in the \textit{duo pollen} mutants. Initial assembly of the \textit{Arabidopsis} MGU in the wild type pollen is apparent at PMII and is completed only at the end of pollen maturation (Lalanne and Twell, 2002).

\textbf{4.3 Two membranes surrounding the generative cell reveal that mutant \textit{duo} pollen grains are bicellular}

An intriguing question was whether the \textit{duo} mutants are binucleate or bicellular. To address this question ultrastructural analysis by transmission electron microscopy was carried out on all three \textit{duo} mutants and was compared to wild type. Two bud stages were selected for comparison, bicellular stage and mature undehisced stage. In wild type at bicellular stage, the GC occupies a cortical position in the vegetative
Figure 4.1: Morphology (light panel) and nuclear constitution of mature *duo3* pollen grains.

A) *duo3* pollen mutants under light microscope; B) DAPI-stained pollen viewed under epifluorescence microscope revealed 50% tricellular pollen grains (yellow arrows) and 50% bicellular pollen grains (green arrows). Scale bar represents 25 µM
Figure 4.2: Nuclear constitution of wild type, *duo1*, *duo2* and *duo3* pollen grains. 
A) Nuclear constitution of wild type pollen grain showing two sperm nuclei (SN) in close association with the vegetative nucleus (VN).
B) and C) Nuclear constitution of *duo2* and *duo3* pollen grains with round generative nuclei (GN) with local chromatin condensation (white arrows)
D) and E) show mutant generative nuclei of *duo1* with discernible groups of chromosomes (white arrows)
cytoplasm. The callose wall is completely degraded and two closely associated plasma membranes surround the internalised GC (Park and Twell, 2001).

Examination of sections (n=30 for each mutant) from bicellular stage in duo1, duo2 and duo3 did not reveal any developmental abnormalities. A double-layered membrane surrounded the generative cell. Cytoplasmic constituents of the vegetative cell, vacuoles, amyloplasts and internal membranes were similar in wild type and duo mutants. Ultra-thin sections from mature undehisced stage were observed for all three mutants (duo1, duo2 and duo3) and compared to wild type. At mature undehisced stage, GC division was completed to form the two SCs in wild type. Elongated profiles with irregular membranes protrusions defined the SCs in the wild type at this stage (Figure 4.3 A, B).

In heterozygous duo1, duo2 and duo3 approximately 50% of the pollen population exhibited mutant phenotype and the remainder of the population was wild type. In the ultrathin sections it was difficult to distinguish between SCs and the mutant GCs. The SCs were not always in paired configuration in the sections. Sections were taken in different planes and they passed through either both or a single SC and some sections did not contain any male gametic cells. Moreover, every section did not run through the middle of the mutant GC. To differentiate between the SCs and the mutant GCs, all the sections that passed through the nuclei were measured using the stage graticule of the electron microscope. The stage graticule provided a rough estimate of the size of the male gametic cells and these measurements were used to distinguish between the sperm cells and the generative cells. The diameters of the nuclei from all the male gametic cells were measured. Mutant GCs could be identified based on their larger diameter (13-30 units) and their distinct regular outline.

In all the duo mutants two intact membranes around the GCs confirmed that mutant pollen grains were bicellular (Figure 4.3 D, F, H). Individual nuclei in mutant GCs were surrounded by an intact nuclear envelope (Figure 4.3 D, F, H). Generative nuclei in duo1 exhibited distinctly different morphology from duo2 and duo3, the chromatin was more condensed (n = 19) (Figure 4.3 G, H). In all duo mutants the vegetative cytoplasm
contains many small vacuoles and clusters of starch granules suggesting that maturation of the vegetative cytoplasm occurs similar to the wild type (Figure 4.3 A, C, E, G).
Figure 4.3: Ultrastructure of wild type, *duo1*, *duo2* and *duo3* at mature undehisced stage

(A) Mature wild type pollen showing vegetative nuclei (VN) and two sperm cells indicated by arrowheads; (B) Two sperm cells in wild type with the vegetative nuclei (VN); (C) Mature *duo2 pollen* mutant showing undivided generative cell (GC) and normal looking vegetative cytoplasm; (D) shows generative nuclei (GN) in *duo2*, cell membrane is indicated by black arrow head and nuclear membrane is indicated by white arrow head; (E) Mature *duo3 pollen* mutant showing undivided generative cell (GC) and normal looking vegetative cytoplasm. (F) The generative nuclei (GN) of *duo2 pollen* mutant with black arrowheads indicating the plasma-membrane and white arrow heads showing the nuclear envelope; (G) shows condensed chromatin structures in *duo1* pollen mutants indicated by white arrowheads; (H) shows double layered plasma-membrane (black arrow head) around the mutant generative nuclei in *duo1* and nuclear membrane is indicated by white arrow head.

Scale bars in (A), (B), (C), (D), (E) and (F) represents 1.4 μm; scale bar in (G) represents 0.5 μm; scale bar in (H) represents 0.23 μm.
4.4 Cellular integrity and *in vitro* pollen germination is normal *duo1*- *duo3*

Cellular integrity and *in vitro* pollen tube germination were monitored to assess pollen viability in *duo* pollen mutants. Mature pollen grains from heterozygous *duo1*, *duo2*, *duo3* and wild type plants were stained with the vital stain, fluorescein diacetate. Vital staining for plasma membrane integrity showed that in wild type approximately 99% of the pollen grains were viable and in *duo1*, *duo2* and *duo3* approximately 97% (n = 400) of mature pollen grains were viable (Table 4.1).

To find out whether the *duo* mutants can form pollen tubes, wild type and *duo* pollen grains were germinated *in vitro*. Germination efficiency in the *duo* mutants was equivalent to wild type (n > 200 for each mutant) (Figure 4.4 A, B). Germinated tubes from the *duo* plants were stained with DAPI solution (Figure 4.4 C) and the number of germinated wild type and mutant pollen grains was scored. In *duo1*, 41% of mutant pollen formed a tube, in *duo2* and *duo3* about 48% of the mutant pollen grains germinated to form a tube.

4.5 *duo* pollen mutants correctly activate vegetative cell marker

To find out whether the vegetative cell (VC) maintains its integrity in the absence of GC division, the nuclear-targeted vegetative cell-specific marker *lat52-gus/nia* was introduced into *duo1*, *duo2* and *duo3*. The *LAT52* promoter has been shown to direct pollen-specific reporter gene expression in *Arabidopsis* and is transcribed specifically in the vegetative cell but not in the generative cell (Twell, 1992). Gene expression in the vegetative cell was directly tested by analysis of GUS activity. Heterozygous *duo1*, *duo2* and *duo3* were crossed to transgenic lines homozygous for the *lat52-gus/nia* transgene in the Nossen background and the F1 progeny was screened for both nuclear-targeted GUS staining and the *duo* phenotype.

The vegetative cytoplasm in *duo1* showed GUS staining and the GC correctly failed to activate the VC-specific transgene. Similarly, in *duo2* and *duo3* the *lat52-gus/nia*
Table 4.1: Percentage viable pollen in wild type, *duo1*, *duo2* and *duo3*

Pollen viability was tested using the vital stain, fluorescein diacetate (FDA). Viable pollen appear green whereas non-viable appear red under UV fluorescence.
Figure 4.4: Pollen germination in wild type and in duo2
(A) Pollen germination in wild type pollen. (B) Pollen germination in duo2. (C) DAPI-stained pollen tubes showing two sperm nuclei (SN) in a wild type pollen tube, and the vegetative nucleus (VN) and single generative nucleus (GN) in germinated duo2 pollen grains.
Figure 4.5 Expression of *lat52-gus/nia* in wild type and *duo3* pollen
A) illustrates wild type pollen grains and *duo3* pollen mutant expressing *lat52-gus/nia* vegetative cell-fate marker and (B) shows the corresponding DAPI picture.
SCs- sperm cells, VN- vegetative nucleus, SN- Sperm nuclei
transgene was expressed only in the vegetative cytoplasm (Figure 4.5). This result indicates that the vegetative cell identity is unaltered and the mutant GCs correctly fail to activate the VC-specific transgene.

4.6 duo mutants deviate from wild type developmental pathway at early tricellular stage

To determine the step at which duo mutants deviated from the normal pathway of pollen development, pollen grains from both wild type and duo plants were examined from successive bud stages. Bud stages were established based on their arrangement on the floral axis (section 2.13.1). Single anthers were dissected; pollen grains were released in DAPI solution and examined by epifluorescence microscopy. No abnormalities were observed during microspore development. In all three duo mutants asymmetric division at PMI and engulfment of the GC into the vegetative cytoplasm were indistinguishable from the wild type.

At -6 stage, in both wild type and duo mutants the pollen population was highly synchronous and was represented by bicellular pollen grains. In wild type -5 and -4 bud stages were not synchronous and pollen grains at different phases of mitosis were observed along with bicellular and tricellular pollen grains. The percentage of tricellular pollen was used as a measure of developmental stage. In wild type approximately 24% and 75% of tricellular pollen grains were observed at -5 and -4 stages respectively and in the succeeding ontogenic stages 100% of the pollen population were tricellular.

However, in duo1 a significant reduction of tricellular pollen grains were observed at -5 and -4 stages. Only 14% and 51% of population was tricellular at -5 and -4 stages respectively. In the following developmental bud stages a maximum of only 53% of the population were tricellular pollen grains in duo1. Similarly, in duo2 the percentage of tricellular pollen grains was reduced to 16% and 49% at -5 and -4 respectively. In duo3 the percentage of tricellular pollen showed a similar trend as the duo1 and duo2. The results showed that developmental programs associated with the formation of the GC in
duo mutants were similar to wild type up to -6 stage and duo1, duo2 and duo3 deviated from the wild type development at -5 stage (Figure 4.6)

4.7 Progression of the generative cell into M phase of the cell cycle

In order to understand the defects associated with generative cell division in the duo mutants, the cell cycle of the male germ line was analysed in wild type and all three duo mutants. In Arabidopsis, analysis of consecutive bud stages provides an excellent system to study progression through cell cycle. It was observed that PMII did not occur strictly at -5 stage (section 4.6) but was extended to -4 stage. The state of pollen grains before PMII (-6 bud stage), during (-5 and -4 bud stage) and after PMII (-3 bud stage) was first analysed in wild type plants.

A single anther was dissected and was examined after DAPI-staining. A homogeneous bicellular population was observed at -6 stage and it was presumed that the generative cells were at G2 phase of the cell cycle. In the next bud stages (-5 and -4 stage) there was a transition from G2 to M phase of the GC cycle. In Arabidopsis, PMII is not truly synchronous in single anthers for any given stage of the cell cycle and generative nuclei with different nuclear morphologies were observed. The rounded generative nuclei underwent morphogenesis to acquire an elongated shape that marked entry into mitosis. Following elongation the generative nuclei underwent M phase of the cell cycle to form the two sperm cells. Therefore, four distinct classes of pollen grain were observed, namely pollen grains with 'a rounded generative nucleus', 'an elongated generative nucleus', 'the generative nucleus in mitosis' and 'two sperm cells'. It was found that the different classes of pollen grains observed at -5 and -4 stages varied in proportion from inflorescence to inflorescence (Figure 4.7 A). In the subsequent stage (-3 stage) a uniform population was observed, sperm nuclei underwent morphogenesis to acquire a spindle shape.

It was reasoned that if the duo mutants were arrested before PMII, a 50% reduction in the number of pollen with elongated generative nuclei and pollen with generative
Figure 4.6: Percentage tricellular pollen grains in the wild type, \textit{duo1}, \textit{duo2} and \textit{duo3}

Graph showing percentage tricellular pollen grains at different stages of pollen development in wild type and in \textit{duo1}, \textit{duo2} and \textit{duo3}. The green box indicates the stages at which pollen mitosis II was observed.
Figure 4.7: Graphs showing the frequency of the different types of pollen grains observed at different bud stages

Graphs showing the frequency of the different classes of pollen observed at bud stages -6, -5, -4 and -3 in (A), (B), (C) and (D). A) In wild type a uniform population was observed at -6 and -3 and at -5 and -4 different classes of pollen grains with different frequencies were observed; B) In duo1, a uniform population was observed at -6. The stages -5 and -4 showed a similar trend to the wild type and at -3 two classes of pollen grains segregating 1:1 were observed; C) and D) represents duo2 and duo3. In both duo2 and duo3 uniform population was observed at -6. At -5 and -4 stages only half of the population showed elongated generative nuclei (GN), GN at mitosis and two sperm cells and at -3 stage two classes of pollen grains segregating 1:1 were observed.
nuclei in mitosis would be predicted. At -6, -5 and -4 bud stages \textit{duo1} followed the same developmental pattern as wild type. Furthermore, the proportion of generative nuclei undergoing elongation and PMII in \textit{duo1} was similar to wild type. However, at -3 stage the ratio of wild type to mutant pollen grains segregated 1:1 (Figure 4.7 B). Our data showed that the GN in \textit{duo1} underwent elongation, entered mitosis but failed to form two sperm cells.

The bud stages -6 to -3 in \textit{duo2} and \textit{duo3} were analysed to establish whether mutant GN elongated and entered mitosis. A homogeneous population was detected at -6 stage in \textit{duo2} and \textit{duo3} as in wild type. In succeeding ontogenic stage the proportion of elongated generative nuclei was significantly reduced, only half of the population underwent nuclear elongation and completed PMII. An equal proportion of wild type and mutant pollen grains were observed at -3 stage (Figure 4.7 C, D). Therefore generative nuclei of \textit{duo2} and \textit{duo3} did not change shape and did not proceed into mitosis.

\section*{4.8 Pollen mitosis II in wild type}

Developmental analysis revealed that \textit{duo1} enters PMII, which is initiated following elongation of the generative nucleus. To establish at which phase of mitosis \textit{duo1} failed, the sequence of events during M phase of PMII was first determined in wild type pollen and then compared to \textit{duo1} pollen mutant. Elongation of the generative nucleus marked entry into mitosis (Figure 4.8 A, B).

At the early prophase, the chromatin in the generative nuclei condensed into five distinct threads like structures (Figure 4.8 C). At late prophase further condensation of the chromatin led to shortening of the chromosomes into five compact chromosomes (Figure 4.8 D). Highly condensed chromosomes congressed on a plane were scored as metaphase (Figure 4.8 E) and two groups of chromosomes opposite each other were scored as anaphase (Figure 4.8 F, G). At telophase two distinct sets of congregated chromosomes segregated from each other leading to the formation of nascent sperm cells (Figure 4.8 H). Newly formed sperm nuclei were rounded and
Figure 4.8: Fluorescence micrographs of pollen mitosis II in wild type
A) and B) show pollen grains at early prophase with an elongated generative
nucleus (GN). C) and D) late prophase with chromatin condensation in the
GN. E) GN at metaphase. F) anaphase with two sets of chromatin. G) and
H) telophase of pollen mitosis II. I) newly formed sperm cells. Scale bar
represents 15 um.
Figure 4.9: Graph showing the mean mitotic index in wild type, \textit{duo1}, \textit{duo2} and \textit{duo3}.

Graph showing the proportion of mitotic figures (early prophase, late prophase, metaphase and anaphase) observed in wild type, \textit{duo1}, \textit{duo2} and \textit{duo3}.

In \textit{duo1} the mean number of pollen grains at early prophase and late prophase was significantly higher than in wild type. In \textit{duo2} and \textit{duo3} the proportion of early prophase and late prophase was reduced by half compared to wild type.

The mean number of metaphase and anaphase mitotic figures was reduced by almost half in all three \textit{duo} mutants.
chromatin was decondensed (Figure 4.8 G). Sperm cells subsequently acquired a spindle shape at anthesis. Therefore in wild type discrete types of mitotic figures at early prophase, late prophase (prometaphase), metaphase and anaphase were counted. The mitotic figures were counted from -5 and -4 stages only and mean mitotic index was 10% (n= 3000) (Figure 4.9).

4.8.1 Pollen mitosis II in the duo mutants

In duo1 prior to PMII the pollen population appeared identical to wild type. Generative nuclei underwent morphogenesis to acquire an elongated shape (Fig. 4.10 B) and chromatin condensation patterns were as in wild type. At late prophase, the chromosomes congressed, but failed to align correctly (Figure 4.10 C, D). Misalignment seemed to lead to excessive condensation (Figure 4.10 E) and the chromosomes appeared as a tight structure. At -3 stage, mutant generative nuclei in duo1 did not remain in a condensed state but showed decondensation patterns similar to sperm cells (Figure 4.10 F). All the mitotic figures observed at -5 and -4 stages in duo1 were counted and the mean mitotic index equalled to 10.1%. Detailed analysis of the frequency of mitotic figures (n = 3300) showed that pollen grains with prophase and late prophase (prometaphase) figures were higher than in the wild type; presumably the mutant pollen grain spends longer time at these steps of the cell cycle. However, the number of pollen grain showing anaphase was reduced in duo1 compared to wild type (Figure 4.9). It seemed that duo1 entered M phase of the cell cycle and failed at prometaphase hence exit mitosis without undergoing chromatids separation.

To confirm that duo2 and duo3 did not enter mitosis, mitotic indices were counted for both mutants and compared to wild type. In duo2, generative nuclei had the same nuclear morphology (Figure 4.10 G) as the wild type at -6 stage. At PMII stage the generative nuclei in duo2 did not show any difference besides an increase in nuclear fluorescence intensity (Figure 4.10 H). In the succeeding stages the generative nuclei remained unaltered (Figure 4.10 I). Moreover, the mean mitotic index was 3.56% (n = 2500) which was almost half the mitotic index seen in the wild
Figure 4.10: Fluorescence micrographs of pollen mitosis II in the *duo* mutants

(A) to (F) *duo1*; A) GC before PMII; B) GC elongation; C) and D) arrows indicate chromatids failing to arrange on the metaphase plate at PMII; E) and F) After PMII the chromatids recondense.

(G) to (I) *duo2*; G) GC before PMII; H) GC during PMII and I) GC after PMII.

(J) to (L) *duo3*; J) GC before PMII; GC during PMII and L) GC after PMII.
type, indicating that 

\textit{duo2} failed at the G2-M transition of the cell cycle (Figure 4.9). 

\textit{duo3} exhibited the same characteristics as \textit{duo2} (Figure J, K, L) and the mitotic index was determined to be 4.23% (n=3200) (Figure 4.9).

\textbf{4.9 Nuclear DNA content measurement}

In all three mutants the generative nucleus was intensely stained with DAPI, indicating that the generative nucleus has undergone DNA replication but failed to divide. This visual impression was confirmed by measuring the nuclear DNA amounts. A technique was developed (described in section 2.13) to measure the nuclear DNA content in GC and SC using DAPI staining. DAPI-stained pollen grains fluoresce in proportion to DNA content (Coleman and Goff, 1985). The nuclear DNA content was analysed in generative and sperm cells from distinct developmental stages. Developmental events associated with the GC and SC is closely related to bud stages. Nine sequential buds were selected based on their arrangement on the inflorescence axis and the stage of generative cell and sperm cell development were defined in wild type and in \textit{duo} mutants.

\textbf{4.9.1 Stages of generative cell and sperm cell development}

At -9 stage unicellular microspores undergo PMI to form binucleate pollen grains; pollen grains at anaphase and telophase were selected for the DNA measurement and were termed incipient generative cells. At -8 stage the GC was on the pollen wall and termed as GC early interphase. At -7 stage the GC was engulfed in the VC cytoplasm and therefore named as GC at mid-interphase. At -6 stage the generative cell appeared more condensed and was completely engulfed in the vegetative nucleus, therefore termed as GC late interphase. PMII occurred predominantly at -5 and -4 stages. Pollen grains at anaphase and telophase were selected and were defined as incipient sperm cells. At -3 stage the sperm cells with rounded profile were classed as sperm at interphase. At -2 and -1 stage the sperm cells had an elongated profile and were termed as sperm prior to dehiscence. At +1, pollen grains were shed, hence were defined as sperm at anthesis. In \textit{duo} mutants, GC ontogeny from -9 to -6 stages was defined as in
the wild type. At stages -5 and -4 the population was asynchronous and mutant GC could not be distinguished from wild type GC. At -3 stage the undivided GC was defined as GC after PMII and the stages -2 and -1 the GC was referred to as GC prior to dehiscence.

4.9.2 Cell cycle pattern in the male gametic cells

The pattern of S phase in the generative cell and the sperm cell was monitored in wild type. Immediately after PMI, DNA content in the incipient generative cell was 1C. At early interphase and mid interphase the DNA content was 1.04C and 1.74C respectively. The GC at late interphase produced fluorescence unit that corresponded to 1.95C. Incipient sperm cells produced DNA content value of 1C and sperm cells at interphase had a C value of 1.09C that increased to 1.20C at anthesis (Figure 4.11). One-tailed t-test assuming unequal variances was performed on these data to compare the DNA content at each ontogenic stage. The generative nucleus entered S phase of the cell cycle soon after its formation, indicating that G1 phase of the generative cell cycle is almost non-existant or very brief as in the sperm cells.

4.9.3 Mutant GCs in duo1 successfully completes S phase but mutant GCs in duo2 and duo3 continues S phase

Relative DNA content was measured in duo1, duo2 and duo3. In duo1, incipient generative cells produced fluorescence units corresponding to approximately 1C. At early interphase GCs in duo1 produced mean fluorescence values that corresponded to 1.06C. The DNA content of GC at late interphase increased to 1.63C and prior to PMII the generative nuclei had DNA content of 1.97C (Table4.2). Maturation of the GC in duo1, in terms of its progression through the S phase, was maintained as in the wild type. The DNA content in the duo1 remained constant following PMII until anthesis (Figure 4.11).

In duo2, the DNA content in incipient generative nuclei was also approximately 1C and the DNA content increased to reach 2C at late interphase. The DNA content
corresponded to 2.31C after PMII. The DNA content was significantly greater than 2C DNA value compared to the generative cell just before PMII. Moreover, the DNA content prior to anthesis was further elevated to 2.46C (Table 4.2). This result showed that the generative nuclei in \textit{duo2} continued S phase of the cell cycle (Figure 4.11). This was suggestive that after PMII the GC in \textit{duo2 pollen} mutant was fated as the sperm cells. In \textit{duo3}, incipient generative cells produced fluorescence values that corresponded to 0.94C. At early interphase GC produced 1.04C DNA content and the DNA content increased progressively to reach 1.98C at late interphase (Table 4.2). Similar to \textit{duo2} there was a significant increase in the DNA content in the generative after PMII. At anthesis, the generative nuclei produced fluorescence value that corresponded to 2.42C (Figure 4.11).

Table 4.2: DNA content in GC and SC in wild type, \textit{duo1}, \textit{duo2} and \textit{duo3}.

s = standard error, C = DNA content.

<table>
<thead>
<tr>
<th>Wild Type</th>
<th>n</th>
<th>mean ± se</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC at telophase</td>
<td>5</td>
<td>18.40 ± 1.17</td>
<td>1.00 C</td>
</tr>
<tr>
<td>GC early interphase</td>
<td>24</td>
<td>19.25 ± 0.69</td>
<td>1.04 C</td>
</tr>
<tr>
<td>GC mid interphase</td>
<td>12</td>
<td>31.94 ± 0.949</td>
<td>1.74 C</td>
</tr>
<tr>
<td>GC late interphase</td>
<td>41</td>
<td>35.76 ± 0.58</td>
<td>1.95 C</td>
</tr>
<tr>
<td>Newly formed</td>
<td>30</td>
<td>18.36 ± 0.38</td>
<td>1.00 C</td>
</tr>
<tr>
<td>SC Interphase</td>
<td>31</td>
<td>20.12 ± 0.58</td>
<td>1.09 C</td>
</tr>
<tr>
<td>Prior to dehiscence</td>
<td>52</td>
<td>21.88 ± 0.38</td>
<td>1.19 C</td>
</tr>
<tr>
<td>Anthesis</td>
<td>36</td>
<td>22.12 ± 0.44</td>
<td>1.20 C</td>
</tr>
<tr>
<td>\textit{duo1}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC at telophase</td>
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<td>18.30 ± 1.12</td>
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<td>GC early interphase</td>
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<td>19.53 ± 0.53</td>
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<td>GC mid interphase</td>
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<td>29.92 ± 1.07</td>
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<tr>
<td>GC late interphase</td>
<td>41</td>
<td>36.24 ± 1.12</td>
<td>1.97 C</td>
</tr>
<tr>
<td>GC after PMII</td>
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<td>36.23 ± 0.99</td>
<td>1.97 C</td>
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<tr>
<td>Prior to dehiscence</td>
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<td>36.50 ± 0.60</td>
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<td>\textit{duo2}</td>
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<td></td>
<td></td>
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<td>GC at telophase</td>
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<td>0.94 C</td>
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<tr>
<td>GC early interphase</td>
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<td>19.21 ± 0.58</td>
<td>1.04 C</td>
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<td>30.80 ± 1.67</td>
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<tr>
<td>GC late interphase</td>
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<td>2.00 C</td>
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<tr>
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<tr>
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<td>1.56 C</td>
</tr>
<tr>
<td>GC late interphase</td>
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<td>1.98 C</td>
</tr>
<tr>
<td>GC after PMII</td>
<td>15</td>
<td>43.03 ± 1.33</td>
<td>2.35 C</td>
</tr>
<tr>
<td>Prior to dehiscence</td>
<td>38</td>
<td>44.60 ± 1.51</td>
<td>2.42 C</td>
</tr>
</tbody>
</table>

94
Figure 4.11: Graphs showing DNA content in the male gametic cells in the wild type, duo1, duo2 and duo3.
A) DNA content in the generative cell and the sperm cell in wild type
B) DNA content in the newly formed GC and in mutant GCs in duo1 after PMII. DNA content remains constant at 2C after PMII.
C) and D) in duo2 and duo3, the DNA content increase progressively after PMII.
4.10 Discussion

4.10.1 Pollen mitosis I is normal in the duo pollen mutants

The most obvious defect in the duo mutants was the inability to undergo generative cell division. The result in section 4.6 showed that in the mutants developmental events before PMII were orchestrated as in the wild type and the earliest defect was only seen at PMII. The ability of duo1, duo2 and duo3 to progress through a specialised asymmetric mitotic division (PMI) and the failure to enter or complete PMII suggested that the generative cell division is regulated in a different way from PMI.

Differentiation of the generative cell depends on the division asymmetry at PM1 and positioning of the plane of division results in the distribution of factors (Eady et al, 1995). It has been stated earlier that if the first mitosis in the microspore is normal, subsequent development and differentiation of the male gametic cells usually occurs normally (Tanaka, 1993). It is interesting to note that in the gametophytic mutants such as sidecar pollen mutants PMI results into two twin cells only one of them is competent to divide. In the duo mutants, PMI is normal and presumably polarised signals exist at the generative cell. This suggests that along with the polarised signals a more complex mechanism exist to trigger the generative cell division.

4.10.2 duo1 fails at M phase of the cell cycle whereas duo2 and duo3 fail at G2-M

Quantification of the mitotic index (section 4.8.1) in duo1 revealed an increase in the mitotic index compared to wild type. A more detailed count on the different mitotic phenotype in the duo pollen mutant showed an elevated frequency of prophase and prometaphase figures. This argues that the mutant pollen grains spend longer at prometaphase. The reduced frequency of anaphase figures indicated that the mutant GCs in duo1 failed to initiate chromosome separation. In higher eukaryotes, the progression into mitosis is mediated by mitosis promoting factors (MPF) that consist minimally of cyclin-dependent kinase (Cdk) and a B-type cyclin regulatory subunit (Porceddu et al, 2001; Ohi and Gould, 1999). Cdk activity is modulated by factors such
as plant hormones (Stals et al, 2000), reversible phosphorylation events (Meszaros et al, 2000; Tsakraklides and Solomon, 2002) and by association with Cdk inhibitors or assembly factors (Wang et al, 2000). It is speculated that key cell cycle regulatory proteins or factors that modulate them might be impaired in duo1.

The terminal phenotype of duo1 did not indicate prometaphase arrest very clearly. But, GC chromatin was very condensed and conglomerated (Figure 4.2 D, E). The inability to undergo chromatids separation might have caused the generative nuclei to partially dismantle its prometaphasic state. Or, potentially the GN in duo1 underwent premature condensation and proceeded to prometaphase of the cell cycle. In alfalfa cultured cells treatment with a protein phosphatase inhibitor led to hypercondensation of late prophase chromosomes that could not enter metaphase (Ayaydın et al, 2000). Mutant duo2 and duo3 did not enter mitosis and were arrested at the G2-M transition, presumably in these mutants regulatory proteins that control entry into mitosis are disrupted.

The results in sections 4.7 and 4.9.3 demonstrated that mutant GCs in duo1 were arrested at M phase after successfully replicating its DNA. Higher eukaryotes undergo open mitosis and the nuclear membrane breaks down during mitosis (Gant and Wilson, 1997). A nuclear membrane was still present in the mutant GC in duo1, although duo1 has entered mitosis it failed to break down the nuclear envelope. The precise stage at which nuclear membrane is broken down at PMII in Arabidopsis is not known. In Ornithogalum virens, a bicellular pollen species, the nuclear membrane is broken down at prometaphase of PMII (Charzynska and Cresti, 1993). In tobacco, the nuclear envelope breaks down at the beginning of prometaphase of PMII (Yu and Rusell, 1993). In Caenorhabditis elegans, the nuclear envelope disassembles very late compared to vertebrates and in Drosophila, full disassembly is seen only during mid-late anaphase (Lee et al., 2000). It is possible that GCs in duo1 were unable to dismantle the nuclear membrane and therefore failed to complete PMII.

4.10.3 S-phase of the cell cycle in the male gametic cells
The synthesis phase of the cell cycle in the sperm cells and generative cells was monitored. In an earlier report it was shown that *Arabidopsis* sperm cells enter S phase immediately after inception (Friedman, 1999). Similar results were obtained using a different technique. Measurements of DNA content in the sperm nuclei revealed that sperm enter S phase soon after its formation and continued DNA synthesis throughout pollen maturation (section 4.9.2). In wild type, DNA measurements revealed that the generative cell enters S phase soon after its detachment from pollen wall. From this data it was speculated that the male germ line follows a S-M phase of the cell cycle. It is interesting to note that in the gametophytic mutant, *limpet pollen* (*lip*) mutant the generative cells remains in close association with the pollen wall and divides to form the twin sperm cells while still attached to the wall (Howden et al., 1998). Presumably, DNA synthesis is not strictly dependent on complete engulfment of the generative cell by the vegetative cytoplasm.

In all three *duo* mutants it was found that the DNA content of the GC reached 2C just before PMII suggesting that mutant GCs completed S phase (section 4.9.3). In plants progression from G1 to S phase requires the concerted action of cyclin-dependent kinase (CDK)-cyclin complexes on specific targets such as the retinoblastoma (RBR)/E2F pathways (Gutierrez et al., 2002). The coordination of DNA replication within the GCs was maintained, this indicates that molecular programme required for replication was functional in *duo* mutants. At anthesis, DNA content remained at 2C in *duol*. In marked contrast, in *duo2* S phase continued and DNA content reached 2.5C at anthesis (Fig. 4.11). It is speculated that DNA synthesis licensing controls operate in *duo2* but failure to enter M phase allow GC to re-enter S phase of the cell cycle just like the sperm cells. In endoreduplicating cells after the completion of S phase, cyclin/CDK activity must fall to low levels before reinitiation of DNA replication is possible. Low CDK activity during G1 allows the assembly of prereplication complexes (preRCs) licensing DNA for another round of replication (Donaldson and Blow, 1999). It has been shown in sea urchins that inhibiting proteosome activity leads to overreplication of DNA and failure to enter mitosis (Kawahara et al., 2000). Mutation in a regulatory proteosomal subunit in *S. cerevisae* causes interphase cell cycle arrest along with overreplication of both nuclear and mitochondrial DNA (Rinaldi et al., 1998). It is
predicted that S phase of the cell cycle are switched off during PMII and immediately after completion of PMII molecular controls of S phase are reactivated.

Isolation of the duo pollen mutants from the morphological screen has proven to be valulable in understanding the events and genetic controls of pollen mitosis II. This study has provided new insights into understanding the developmental programs associated with the S phase of the generative cell and sperm cell. It is speculated that molecular controls of S phase are initiated in the generative cell and at PMII mitotic cyclins/cdk activity increases which switches off the S phase cyclin/cdk activity therefore turning off the synthesis phase. Upon completion of PMII, sperm cells are formed that undergo S phase immediately after inception. The exit from mitosis triggers decrease in levels mitotic of cyclins and S phase cyclins are reactivated, leading to DNA synthesis. The distinctive phenotypes and strong penetrance of duo1, duo2 and duo3 mutations will facilitate cloning of their respective genes to reveal their precise roles in the generative cell division cycle.
Chapter Five

Isolation of the *DUO3* gene
Chapter 5
Isolation of the DUO3 gene

5.1 Introduction

Morphological screening of 10,000 EMS-mutagenised plants led to the identification of a unique class of pollen mutant termed the duo pollen mutants (Park et al., 1998). Out of the six duo mutants identified, duo3 was selected for map-based cloning. duo3 shares similar phenotype as duo2 and it is highly penetrant. Moreover, genetic analysis revealed that duo3 is a male specific gametophytic mutation. It was already established that duo3 is linked to the marker nga280 on the lower arm of chromosome 1 (section 3.6.3).

In this chapter the molecular mapping of DUO3 is presented. New molecular markers were developed and were exploited to identify recombinants in the proximity of the DUO3 gene. The genetic interval containing the duo locus was narrowed down to a 10 kb region containing two genes. Functional complementation was carried out by introducing a wild type copy of the gene into heterozygous duo3 and the transgenic plants were analysed for reversion to wild type phenotype. The outcome of the complementation provided a definite proof of the DUO3 gene. Expression of DUO3 in the wild type was confirmed using RT-PCR and bioinformatics searches were performed to understand the nature of the novel gene.

5.2 Mapping population

A mapping population was created by outcrossing heterozygous duo3 plants (Nossen) (No-0) as female to wild type Columbia (Col-0) plant. Initially 80 F2 plants were used to establish linkage and the chromosomal location for duo3 mutation was defined on chromosome 1 (section 3.6.3). Mapping resolution is mainly determined by the size of the mapping population (Lukowitz et al., 2000). Two different mapping populations
were analysed to map \textit{duo3}. Approximately 650 F2 plants from selfed F1 (T61D) plant
were screened for pollen phenotype. The F2 plants segregated into wild type and mutant
plants in the ratio 1:1 and approximately 300 plants bearing the wild type (Col-0/ Col-0)
phenotype were selected for mapping. In the second mapping population approximately
550 F2 plants from a different F1 (T61E) plant were screened for pollen phenotype and
both wild type and mutant plants were selected for mapping.

The \textit{duo3} mutation is in the No-0 background and wild type plants are homozygous for
Col-0 alleles (Col-0/ Col-0) at the \textit{DUO3} locus. The presence of the Col-0 and No-0
allele (Col-0/ No-0) would indicate a single crossover event whereas two No-0 alleles
(No-0/ No-0) would represent double recombination events. However, non-recombinant
\textit{duo3} plants would have C and N alleles (Col-0/ No-0) at the \textit{duo} locus and
recombinants would have either Col-0/ Col-0 or No-0/ No-0 alleles.

5.3 Molecular markers used to isolate \textit{DUO3}

The \textit{duo3} locus was delineated between the two PCR-based markers \textit{F12H21} (91cM)
and \textit{F12A21} (100cM), located on the BAC F15H21 and BAC F12A21 respectively
(section 3.6.3). These markers lie 9 cM apart and new markers were created and
exploited to narrow down the genetic interval containing the \textit{DUO3} gene.

To exploit the north and south recombinants it was necessary to create new molecular
markers in the 9cM region. An extensive collection of Columbia and Landsberg erecta
polymorphic markers are accessible from the TAIR database, but there are very few
markers that show polymorphisms between Col-0 and No-0. TAIR (http://www.arabidopsis.org/cgi-bin/maps/Schrom)
contains information about all the simple-sequence repeats that are longer than 30 nucleotides in length, along with 200 bp
flanking the repeated region. These short repetitive sequences provided a good starting
point to develop SSLP markers. In order to find length polymorphisms between the two
ecotypes new molecular markers were developed using the Col-0 sequence. Both Col-0
and No-0 DNA were amplified with the new markers and the size difference between
the two ecotypes was resolved on 4% agarose gels.
Another strategy used to identify polymorphisms was by sequencing random fragments of DNA (approximately 1 kb fragments) in the region of interest. A pair of oligonucleotide was synthesised for each DNA sequences. Stretches of intergenic regions or large introns were selected to maximise the chances to find polymorphisms and wild type No-0 plants were amplified and sequenced. The sequences were then compared with the existing Col-0 sequence to detect polymorphisms. Simple nucleotide polymorphisms (SNPs), deletions and insertions were identified from the sequencing information. The SNPs and the deletions were used to generate ecotype specific markers (Col-0 or No-0 specific), derived cleaved amplified polymorphic (dCAPS) markers or SSLP markers (Table 5.1).

To create SSLP markers (RH1, W2XF and RABC) primers were designed across deletions or insertions that were greater than 12 bp in length so that the size difference could be visualised on an agarose gel. The product size was maintained between 175 to 200 bp for the SSLP markers. Three or four base pair deletions or insertions were also exploited to create Col-0 specific marker (CDFN7). Ecotype specific markers were created such that the primers contained the insertions or deletion at the 3’ end. SNPs were used to generate dCAPS markers according to Michaels and Amasino, 1998. To create dCAPS markers (DRA5, DN4) a mismatched PCR primer was designed next to the polymorphic position such that an artificial restriction site was created with the sequence variant in one accession but not in the other accession. The PCR products were usually less than 180 bp and the size difference after digest depended on the length of the mismatched primer.
Table 5.1: Molecular markers used to map *DUO3*.

<table>
<thead>
<tr>
<th>Markers</th>
<th>BAC clone</th>
<th>Marker position(bp)</th>
<th>Type</th>
<th>Primer F</th>
<th>Tm °C</th>
<th>Primer R</th>
<th>Tm °C</th>
<th>Product size</th>
</tr>
</thead>
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<td>nga280</td>
<td>F14J16</td>
<td>20939226</td>
<td>SSLP</td>
<td>GGCTCCATAAAAGTGCACC</td>
<td>60</td>
<td>CTGATCTCACGGACAAATATGC</td>
<td>60</td>
<td>Col-0 &gt; No-0</td>
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<td>T7P1</td>
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<td>25494728</td>
<td>SSLP</td>
<td>TCTGGTGCTGACTTCTTTTG</td>
<td>66</td>
<td>GCATTACGGATATGCTTCAA</td>
<td>65</td>
<td>Col-0 &gt; No-0</td>
</tr>
</tbody>
</table>
5.4 Genetic mapping of **DUO3**

The F2 mapping population (821 plants) was screened for recombinants with the north marker *F15H21* (~91 cM) and the south marker *F12A21* (~100 cM) and 139 recombinants were collected using these flanking markers. Map position of *duo3* was established by assessing the relative recombination frequencies with the flanking markers. A recombination frequency (RF) of 1.52 % (25 recombinants) was obtained from the north marker (*F15H21*) and 6.94 % RF (114 recombinants) was obtained with the south marker (*F12A21*).

All the south recombinants (114) were screened with the SSLP marker *F5I14* (~95 cM). Out of 114 south recombinants, 18 samples were still recombinants by the marker *F5I14*. The recombinants with the marker *F15H21* (25 north recombinants) and the recombinants with the marker *F5I14* (18 south recombinants) were analysed with the CAPS marker *g11447* (92 cM). Only one south recombinant was maintained and all the north recombinants were non-recombinants by the marker *g11447*. The *duo* locus was therefore defined on three BACs *F15H21*, *F1N19* and *F13011* (Figure 5.1).

Six markers (RRA1, RH1, DRA5, W2XF, RABC and DN4) were designed on the BAC *F1N19* and the recombinants were tested. The recombinants with the marker *F15H21* (25 north recombinants) were exploited with the marker *DRA5* on the BAC *F1N19* and four of them were still recombinants (Figure 5.1). Therefore, *DUO3* locus was further narrowed down on the overlapping BACs *F1N19* and *F13011*.

The four north recombinants (with *DRA5*) were tested with the markers W2XF and RABC and only one sample was still recombinant with the marker RABC. Only one south recombinant was recovered with the marker *g11447*. The south recombinant was tested with the markers CDFN7 and DN4 and it was consistently scored as a recombinant with these markers (Figure 5.1). Therefore, the *DUO3* locus was
Figure 5.1: Schematic representation of map-based cloning of DUO3 gene

A) Small arrows indicate the positions of the markers and the numbers in italics represent the number of recombinants. DUO3 locus was defined on two BACs F1N19 and F13O13 between the north marker DRA5 and the south marker g11447. Markers were created on the BAC F1N19 and the duo3 locus was delineated to a region containing two genes. B) Gel electrophoresis of PCR products from a linked marker and unlinked marker and the recombinants obtained with the marker g11447(C: columbia alleles; N: nossen alleles; H: heterozygote)
delineated to a 10 kb region containing two genes and all the recombinants were fully exploited in the region. Details of DUO3 mapping has been included in the appendix.

5.5 Sequencing the two putative candidates

The DUO3 locus was defined to a 10 kb region containing two genes, At1g64570 and At1g64580. Both genes in the duo locus were identified as hypothetical proteins. The ORF of At1g64570 gene, determined by conceptual translation, encodes a putative protein of 1239 amino acids with a molecular mass of 137 kDa. This gene has eight exons interrupted by seven introns. Blast searches revealed that the gene At1g64570 exists as a single copy in the Arabidopsis genome. The ORF of the At1g64580 gene encodes a putative protein of 1052 amino acids with molecular mass of 119 kDa. The gene has two exons interrupted by an intron. Blast searches revealed 52% identity with the Arabidopsis gene At5g41170 that is annotated similar to Nicotiana tabacum salt inducible protein. Nine sets of primers were designed to amplify the entire duo3 locus (10 kb). Genomic DNA from heterozygous duo3 plants was sequenced but revealed no point mutations in the DUO3 locus that could cause truncation of the protein.

5.6 Complementation

Complementation is the most conclusive method of proving that the gene of interest causes the mutation. It was established that duo3 is a recessive mutation (section 3.5) complementation test could be carried on the loss-of-function mutation. For the complementation of duo3 two parallel strategies were followed. A cosmid library (CD4-11, Arabidopsis Biological Resource Center) containing approximately ten genomic equivalents of Arabidopsis wild type DNA (ecotype: wassilewskija) was screened to identify clones containing the genes of interest. Two cosmids clones containing the desired At1g64570 gene and a single cosmid clone containing the gene At1g64580 was identified. Overlapping clones were also identified and mobilised into Agrobacterium, however the construct proved to be very unstable in Agrobacterium resulting in lost of the construct.
Chapter Five

Isolation of the DUO3 gene

As an alternative approach, the ORFs of the gene At1g54570 and the gene At1g64580 were cloned by PCR from wild type genomic DNA and were inserted into heterozygous duo3 by the floral dip method (Clough and Bent, 1998). The genomic region of the gene including 1 kb upstream and 1 kb downstream was amplified using a proofreading polymerase (pfu) in order to minimise the effect of a PCR amplification errors. Unique restriction sites were included in the primers that were used to amplify fragments. The vector used was pPZP221 (Hajdukiewicz et al., 1994) that confers resistance to spectinomycin in bacteria and resistance to gentamycin in plants.

5.6.1 Construction for the pPZNTS and pPZSAT plasmids

The gene At1g64580 was cloned into the vector pPZP221 to form pPZNTS. A 5.7 kb fragment including the genomic region of the gene At1g64580 and 1 kb upstream and 1 kb downstream the gene was amplified by PCR. Two oligonucleotides primers including a KpnI and a BamHI sites at the 5' and 3' ends respectively were used for the PCR amplification. The PCR product as well as the vector pPZP221 was digested with the restriction enzymes KpnI and BamHI. The cleaved PCR product was then ligated into the digested pPZP221 vector (Figure 5.2).

A second construct (pPZSAT) harbouring the gene At1g64570 was made to transform heterozygous duo3 plants. A 7 kb fragment containing the genomic region of the gene At1g64570, including one kb flanking the gene was amplified by PCR. Two oligonucleotide primers with a KpnI and an XbaI sites at the 5’ and 3’ ends respectively were used to amplify the At1g64570 gene. The PCR amplified fragment and the vector pPZP221 was digested with the same set of restriction enzymes. Following restriction digestion the PCR product and the vector were ligated (Figure 5.3).
Figure 5.2: Plasmid construct pPZNTS
Two oligonucleotide primers (5' - tggaggtacctatgaacgaggtttggtcacttcg-3' and 5' - tgaaggatccaaacccctgttccacaagaagacc-3') containing Kpnl and BamHI sites respectively were used to amplify a 5.7 kb fragment containing the gene At1g64580. The fragment was cloned into pPZP221.

gentR- gentamycin resistance gene; specR- spectinomycin resistance gene
Figure 5.3: Plasmid Construct of pPZSAT

Two oligonucleotide primers {5'-tccgggtaccaaccaacatactttcaacag-3' and 5'-tccgggtaccaaccaacatactttcaacag-3'} containing KpnI and XbaI sites were used to amplify a 7 kb fragment containing the gene At1g64570. This fragment was cloned into pPZP221.

gentR- gentamycin resistance gene; specR- spectinomycin resistance gene
The ligated DNA for the construction of pPZNTS and pPZSAT were transformed independently into *E. coli* competent cells. Recombinants were screened for blue/white selection (α-complementation). The presence of the inserts was tested by colony PCR and the expected size of the inserts was confirmed by restriction enzymes digestion. Two clones were then selected for each construct to transform *Agrobacterium GV3101* by the freeze-thaw method (Höfgen and Willmitzer, 1998). The presence of the plasmids in *Agrobacterium* were tested by colony PCR and heterozygous duo3 plants were transformed by floral dipping (as described section 2.15.5), allowed to grow to maturity and the TI seeds were collected.

### 5.6.2 Analysis of the transformants harbouring the construct pPZNTS

The T1 seeds (containing the construct pPZNTS) were grown on MS medium with gentamycin selection, 58 primary transformants were recovered and were allowed to grow to maturity. DAPI-stained pollen from the primary transformants were visualised under an epifluorescence microscope to determine the phenotype of the transformants. A ratio of 1:1 for wild type to duo3 plants was obtained. Pollen phenotype of all the duo3 plants (27 plants) was scored. In a fully complemented line the proportion of wild type: duo pollen should revert from 1:1 to 3:1 for an unlinked insertion. All the duo plants showed 50% wild type and 50% duo pollen (two-tailed t-test at 1% level of significance showed evidence that transformants were segregating 1:1 for wild type to duo pollen grains (p-value = 0.014) as expected for duo3 heterozygous parent (Figure 5.4). This result showed that the gene At1g64580 did not complement the duo3 mutation.

### 5.6.3 Analysis of the transformants harbouring the construct pPZSAT

The T1 seeds were grown on MS medium with gentamycin and 60 transformants were recovered from this the construct. The transformants segregated into 24 wild type and 36 duo3 plants. Pollen phenotype of all the 36 duo transformants were screened to check for reversion from 1:1 to 3:1 wild type to mutant pollen phenotype. It was found that the
Figure 5.4: Analysis of transformants harbouring the construct pPZNTS

Graph to show percentage mutant (duo) pollen grains in transformants harbouring the construct pPZNTS. A two-tailed t-test at a 1% level of significance provides evidence that the transformants are segregating 1:1 (p-value=0.014)
Figure 5.5: Analysis of transformants harbouring the construct pPZSAT

A) Graph to show the segregation of mutant pollen grains (duo) in transformants harbouring the complementation construct pPZSAT. Red columns indicate putative complemented lines and blue columns represent parent heterozygous duo3 plants (parental line). A two-tailed t-test at 1% level of significance provides evidence that the transformants are not segregating 1:1 (p-value=0.000)

B) Transformants were classified into three groups based on the proportion of mutant pollen in each transformed line. A two-tailed t-test (assuming equal variances) show that the three samples are statistically different from each other. I v/s II, t stat = 7.44 (p-value = 0.000); I v/s III, t stat = 8.92 (p-value = 0.000); II v/s III, t stat = 5.24 (p-value = 0.001)
transformants were not segregating 1:1 for wild type to duo pollen grains (A two tailed t-test at 1% level of significance showed evidence that transformants were not segregating 1:1, p-value = 0.000) (Figure 5.5A).

The transformants (with duo phenotype) harbouring the construct pPZSAT were classified into three categories based on the proportion of mutant pollen grains from each transformed line. In 17% of the primary transformants the proportion of duo pollen grains ranged from 40 - 44%, 5% of the transformants showed less than 40% (25% or 35%) mutant pollen grains and the remaining transformants showed more than 44% mutant pollen grains. A two-tailed t-test (assuming equal variances) showed that the three samples are statistically different from each other. This result revealed that 17% of the transformants partially complemented duo3 mutation and 5% of the transformants fully complemented duo3 mutation. In partially complemented lines the segregation of wild type to mutant pollen was 1.5: 1 and in fully complemented lines the segregation ratio was 2.6:1. In the non-complemented lines the proportion of duo pollen were similar to duo3 heterozygous parental lines (Figure 5.5A,B)

5.7 Genetic analysis of the revertants containing the construct pPZSAT

The duo3 mutation is not transmitted through the male gametes (transmission efficiency = 0) (section 3.4). However, if the introduced DUO3 gene (At1g64570) has successfully complemented the duo3 mutation, the duo3 mutant allele should be transmitted by the male gametophyte. In the revertants (hemizygous for the T-DNA and heterozygous for duo3) a maximum transmission efficiency (TE) of 33% was expected (Figure 5.6) and 33% TE represented the maximum TE (represents 100%). Pollen grains from the revertants (T1, T2, T3, T4, T5 and T6) were used to pollinate the male sterile plants (MS-1) (Dawson et al., 1993). The seeds resulting from this cross were allowed to grow till maturity and screened for pollen phenotype. In all the complemented lines, male transmission was rescued. The TE varied between 5-55% in the transformed lines (Figure 5.7). The presence of the T-DNA was confirmed by PCR. Non-complemented lines that were used as control did not
A) Heterozygous \textit{duo3} (+/\textit{duo3}) plants produce male gametes carrying the either wild type allele or \textit{duo3}, the gametes carrying \textit{duo3} are not transmitted. Therefore, TE through the male is zero.

B) In double heterozygotes the male gametes carry +; T-DNA(+), +; T-DNA(-), \textit{duo3}; T-DNA(+) and \textit{duo3}; T-DNA(-). However, \textit{duo3}; T-DNA(-) is not transmitted through the male gamete. Therefore the maximum TE of \textit{duo3} mutation in double heterozygotes = 1/3*100= 33%.

TE (transmission efficiency) represents the fraction (%) of mutant alleles that successfully transmit the mutation.
Figure 5.7: Genetic transmission of *duo3* through the male gamete

Graph showing the genetic transmission of *duo3* through the male in the revertants. (T1-T6). Male transmission is rescued and the transmission efficiency (TE) ranged between 5-55 %, in the non-complemented line (C1 as control) there is no transmission through the male.
show transmission through the male partner. This data showed that the transcription unit (At1g64570) represents the DUO3 gene.

Furthermore, the revertants were allowed to self fertilise and the progeny (T2) were grown to maturity. The segregation of the wild type plants and the mutant plants (duo3) among the T2 progeny was scored. It was expected that selfing of double heterozygotes would produce wild type to mutant plants in the ratio of 4:8 (1:2) (Table 5.2). However, in fully complemented lined a segregation ratio of 5:7 (1:1.4) will be produced. The plants with the genotype duo3; duo3/T-DNA(+); T-DNA(+) would have a wild type phenotype if there is 100% rescue. In the revertants T1, T2, T4, T5 and T6 the segregation of wild type to duo plants were 1:1.30, 1:1.28, 1:1.45, 1:1.42 and 1:1.35 respectively (Table 5.3), the Chi-squared test ($\chi^2$) showed that the ratio do not significantly differ from the expected 1:1.4 ratio in the transformed lines (wild type to duo mutant plants), $\chi^2=3.84$ (P=0.05).
Table 5.2: Expected ratio of wild type to duo plants from selfed double heterozygotes

Table shows the expected phenotype (shown bold in brackets) of the progeny produced by selfed double heterozygotes. duo3/ T-DNA(-) is not transmitted through the male gamete. The expected ratio of wild type: duo plant from complemented lines would be 4:8 (1:2). However, in fully complemented lines the segregation of wild type: duo plant would be 5:7 (1:1.4), plants with the genotype duo3; duo3/ T-DNA(+); T-DNA(+) would have a wild type phenotype.
Table 5.3 Complementation analysis of *duo3*

<table>
<thead>
<tr>
<th></th>
<th>Without antibiotic selection</th>
<th>Segregation of T-DNA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Wt</td>
<td><em>duo</em></td>
</tr>
<tr>
<td>T1</td>
<td>23</td>
<td>30</td>
</tr>
<tr>
<td>T2</td>
<td>32</td>
<td>41</td>
</tr>
<tr>
<td>T3</td>
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<td>23</td>
</tr>
<tr>
<td>T4</td>
<td>58</td>
<td>84</td>
</tr>
<tr>
<td>T5</td>
<td>42</td>
<td>60</td>
</tr>
<tr>
<td>T6</td>
<td>40</td>
<td>54</td>
</tr>
</tbody>
</table>

The segregation of wild type and *duo* plants from selfed double heterozygotes (T1-T6)

* The Chi-squared test (\(\chi^2\)) shows that these ratios do not differ significantly from the expected 1:1.4 ratio (wild type to *duo* mutant plants), \(\chi^2 = 3.84\) (P=0.05). § The Chi-squared test (\(\chi^2\)) shows that the ratio differ significantly from the expected 1:1.4 ratio (wild type to *duo* mutant plants), \(\chi^2 = 3.84\) (P=0.05)

TE\(^{\text{male}}\) represents the transmission efficiency through the male gametes in the transformed lines.

All the lines (T1-T6) are segregating 3:1 for resistant to sensitive plants, indicating the presence of a T-DNA at a single locus. * The Chi-squared test (\(\chi^2\)) shows that these ratio do not differ significantly from 3:1 ratio (resistant: sensitive ratio), \(\chi^2 = 3.84\) (P=0.05)
5.8 DUO3 is expressed in all tissues

Total RNA was extracted from the vegetative and the reproductive organs of wild type plants and gene expression pattern was analysed by reverse transcription-PCR (RT-PCR). A pair of primers was designed, the forward RT-PCR primer (SANTF), CTCTTTGTGCACCTTGATCATTACG, was designed in the 1st exon, the reverse RT-PCR primer (SANTR), GCTCGCCTTTACTAGGAAGAAACC lied in the 3rd exon and yielded a product of 611 bp. Expression analysis was carried out using tissues from wild type roots, leaves, stem, flower, pollen and microspores. DUO3 was expressed in all the tissues (Figure 5.8). The Arabidopsis kinase-associated protein phosphatase (KAPP) gene that shows constitutively expression was used as a positive control.

5.9 The DUO3 gene

The Arabidopsis DUO3 gene (DUO3) encodes a predicted protein of 1239 aa with a molecular mass of 137 kDa. According to the annotations provided at TAIR the gene structure consists of eight exons and seven introns organised over a 5 kb genome region (Figure 5.9). One EST (accession: AV554858) is available from the database and it confirms the junction between the first exon and the second exon.

Blast searches (http://www.ncbi.nlm.nih.gov/BLAST/) revealed that DUO3 exists as a single copy in the Arabidopsis genome and the protein does not show similarity with any known protein. However, the Arabidopsis protein (AtDUO3) is related to an unknown protein from Oryza sativa, accession AC116369.5, AAPO3395 (japonica cultivar-group). An alignment of the amino acid sequences of AtDUO3 and the Oryza sativa gene (OsDUO3) revealed 38% identities and 14% similarities (Figure 5.10). OsDUO3 encodes a predicted protein of 1178 aa with a molecular mass of 131 kDa. The exon and intron junctions of the rice gene were available from the database and revealed that the sizes of the exons were similar in the rice and the Arabidopsis gene (Figure 5.9).
Figure 5.8: RT-PCR analysis of DUO3 in wild type
A) shows the control KAPP (kinase-associated protein phosphatase gene) that is constitutively expressed.
B) RT-PCR analysis of DUO3 showing expression in root, leaves, stem, flower, microspore and pollen
Figure 5.9: Structure of the DUO3 gene and the protein from Arabidopsis (AtDUO3) and Oryza sativa (OsDUO3)

A) Structure DUO3. The red boxes indicate exons and black lines, introns. The numbers on top of boxes indicate the size of exons. Both AtDUO3 and OsDUO3 consist of eight exons interrupted by seven introns. One EST (AV554858) is available that confirms the exon1 and exon2 junction.

B) DUO3 peptide sequence in Arabidopsis and Oryza sativa showing the aspartic acid rich (ASP); the SANT domain and the glutamic rich region (GLU). The numbers on top correspond to the amino acid numbers of the region in the protein.
Figure 5.10: Alignment of AtDUO3 and OsDUO3

The alignment of the DUO3 protein from Arabidopsis (AthDUO3) and from Oryza sativa (OsDUO3). Sequences were aligned with CLUSTAL W using default parameters. Identical and similar residues are shown in black and grey background respectively. Gaps required for the optimal alignment are indicated by dashes. The SANT domain present in AthDUO3 and OsDUO3 was determined by motif scan and is indicated by a line below the sequences.
Motif scan (http://hits.isb-sib.ch/cgi-bin/PFSCAN) revealed that *AtDUO3* gene has two glutamic acid-rich region between amino acids 28 to 41 and between amino acids 1078 to 1118, an aspartic acid-rich region (157 to 217 aa) and a SANT domain (501 to 547 aa) (Figure 5.11). The *OsDUO3* gene contains a single glutamic acid-region (1013 to 1049 aa) and a SANT domain (539-693 aa) (Figure 5.12). The *AtDUO3* gene contains a putative nuclear localisation signal (NLS) (http://maple.bioc.columbia.edu/predictNLS/), suggesting that it is localised in the nucleus. SMART (Simple Modular Architecture Research Tool) allowed the identification and annotation of genetically mobile domains and it revealed that *AtDUO3* protein has a SANT domain as predicted by motif scan and has low complexity. Motif scan predicted a single SANT domain (539-693 aa) in the *OsDUO3* protein whereas SMART predicted two SANT domains in the *OsDUO3* protein between the amino acids 483 to 532 and between the amino acids 546 to 605.

The SANT domain is a novel motif that was identified based on its homology to the DNA binding domain (DBD) of c-Myb (Figure 5.13). The Myb-DBD consists of tandem repeats of three α helices in a helix-turn-helix arrangement. Each α helix contains conserved bulky aromatic residues that play a key role in helix packing. These residues are also conserved in the SANT domain, suggesting the tertiary structure of the SANT domain is similar to Myb-DBD (Aasland et al., 1996). The SANT domain is present in number of proteins such as the SWI-SNF, ADA, N-CoR and TFIIBB from which the SANT domain derives its name. Searches using SMART revealed that in *Arabidopsis* about 453 genes contain the SANT motif. It was found that the SANT domain occurs either singly or in multiples. In 208 proteins a single SANT motif was present, 232 proteins contained two SANT domains and 11 proteins had three SANT motifs. One protein with four SANT domains and another protein with five SANT domains were found.
Fig 5.11 Amino acids sequence of AtDUO3 and the motifs predicted by motif scan
Amino acid sequences of AtDUO3 showing the glutamic rich region, the aspartic rich region and the SANT domain. The motifs were predicted by motif scan (http://hits.isb-sib.ch/cgi-bin/PFSCAN)
OsDU03 MEVDDEVEMDIDFNFVLRGSPSETSSLITSEARCRGDSDPDQRRSSSVYPGHGNYMETSACLPSALLSRLPCCRTEN
OsDU03 FESTSSQVPCENMDGCGCNMLQEALPSVEACSSPPLSKDSSHLLEGSZSDACRPRRAVARSSLKNSLELETFLQLQSDDDG
OsDU03 DQVNSDDERESSYKFLAAVLSGDDGTQCACQGDENDEDENDAFLSIEEALESDDGDAENYUTIMREDGDRRQTR
OsDU03 KNPCELSGAANEGVSTKKSLRPLYFISPPELLASGQPYQYWQPSQTIFSPSSIMPVQNGAALANGSDQQLRJMLI
OsDU03 YEHVQLLLTIQFNSLVFSDKQLAIVMDIVELVGGCDDRAALASSTIHQPCCFPQHLSASSFSSETLQYYQ MQLI
OsDU03 SPVMISLDSPLHLAGLYLDVSDTSKQSKQKTTLEALTLENTKESVALVPSDIARNAERFPLFSNLSSLFHPPPITA
OsDU03 MANRVLFTDAEDGGLLLGLLEXYNNQVWGAIKQFILPKCSRQHQIFVPQKNSSSKAPDNPQETVRRMETSPTLNEBQRIQE
OsDU03 GLEAKFSNDWLVVRRPVPHDFPSILLPQMRATSATOVQKSYNKSRAEKEKRRSTEAKRKLKASMPHSQVARQQRADNNGSE
OsDU03 GAENDDDDL YVNEAFLADTESR1NYQVPQLSFLPNAGKQMQGSGSLSCEESGVASDAEQKQONSTNFDVTASAYPFSS
OsDU03 SCTSGLSSRKKVQGGSLQFAQSFSKEKGSVCVKLAFDLFFPNLSPPVIRISQVAFQHTANATQLGTSDNAAKDLFVF
OsDU03 FPPFSERVQNLFFDFHNVRHLQHSGISNGTTEGDAEQDFQFMHLLFQYPREVLSYSHNVQNLLNHSRDLFPFEKV
OsDU03 QTREKSNHTDTCEITRTPVNAINTDPHPLQQTREEMHGEGVPDCNRPYNQSECNMREAPADQSTARKKSTGCPCKEN
OsDU03 NIDLDDILCSRDMNQGTDGSSKLNDRAEQSRKQAXVSELELDGQVCSVHIGIEPEENRSMOQIVMEQQELELSDEEDS
OsDU03 QHFZESREMDSSDEDQVQVGDPLAQKEVSTVGGCQEGSNQNSQHQLQVQGHKQAAGQFQRLSNARPAKXL
OsDU03 KGENAKRPSRTTQBGSSHSTTPPTETSQKTTRPFQAOVQIGAERKKSSDSSRSSKKFAPS

Fig 5.12 Amino acids sequence of OsDUO3 and the motif predicted by motif scan
Amino acid sequences of *oryza sativa* (OsDUO3) (accessionAC116369.5 AAP03395) showing the SANT domain and glutamic acid rich region (http://hits.isb-sib.ch/cgi-bin/PFSCAN)
Figure 5.13: Alignment of SANT domains and MYB domains

Alignment of SANT domains from different proteins is shown. The three repeats of the human cMYB DNA binding domain are likewise shown for comparison.

A consensus common to the SANT domains shows: %, semi-conserved hydrophobicity; #, strongly conserved hydrophobicity; -, conserved acidic residues; +, conserved basic residues (from Aasland et al., 1996).

A secondary structure prediction generated with the program PHD is shown; H, α-helix; E, extended sheet (from Aasland et al., 1996)
5.10 Discussion

5.10.1 Map based cloning approach was used to isolate DUO3.

Two different mapping populations were used for the mapping of DUO3 and both consistently positioned DUO3 to the same chromosomal location. The molecular markers used in this mapping experiment included SSLP, CAPS and dCAPS markers. These markers are co-dominant allowing both chromosomes to be genotyped and allowing maximum information to be gathered from the mapping population. Moreover, the markers are PCR based and can be analysed on agarose gels, which made it easy to use and inexpensive. duo3 showed linkage with the marker nga280 with 15% recombination frequency (section 3.6.3). Markers north (SOLO39) and south (T7P1) of nga280 were exploited and it was established that DUO3 was located south of nga280. New SSLP markers were created and were analysed so that DUO3 was delineated between the markers F15H21 and F12A21. Fine mapping narrowed down the duo3 locus to a 10 kb region containing two hypothetical genes. This mapping resolution was attained using approximately 800 plants (1600 chromosomes). In a number of positional cloning projects a mapping resolutions of 10 to 40 kb have been obtained using a mapping populations of approximately 1000 plants (Ryals et al., 1997, Sakai et al., 1995, Hardtke et al., 1998).

5.10.2 Molecular complementation

A wild type copy of DUO3 introduced into heterozygous duo3 plants fully restored the wild type phenotype. Two contracts pPZSAT and pPZNTS harbouring the gene At1g64570 and the gene At1g64580 respectively were made. The construct pPZNTS did not complement duo3 whereas the construct pPZSAT complemented the duo3 mutation. Male transmission in the revertants was rescued, confirming the identity of the gene. About 17% of the tranformants showed partial complementation, this could be due to several reasons. The site of integration in the genome plays an important role in the level of expression. If the gene becomes integrated in the heterochromatin region, expression will be low. Moreover, sub-optimal regulatory signals, promoters and
enhancers can also reduce expression. Fully complemented lines showed restoration of the mutant phenotype by the wild type copy of the gene. Moreover, male transmission was rescued up to 55% in some revertants. This proved that the transcription unit At1g64570 represents the DUO3 gene.

5.10.3 No base change detected at the duo locus

Genomic sequencing of the open reading frames in the duo locus did not reveal nucleic acid base change that could disrupt the function of the gene. Gene function and expression can be modified by epigenetic factors and not only due to changes in the DNA sequence. SUPERMAN gene and the clark kent (clk) alleles of Arabidopsis provide a well documented example for heritable changes in the gene expression that are not related to differences in the underlying DNA sequences. Sequencing of the SUP coding region from clk alleles revealed no nuclei acid sequence difference from wild type. Whereas the active SUPERMAN allele remains unmethylated, the inactive clk displays significant DNA methylation. The clk alleles could be complemented with a transgene carrying a copy of SUPERMAN (Jacobsen et al., 1997). Similarly, duo3 could be complemented with the wild type copy of DUO3. It remains unclear how irradiation or EMS mutagenesis contributed to the formation of stably inherited epigenetic alleles.

In Arabidopsis two kinds of DNA hypomethylation mutants have been isolated, ddm1 mutants isolated from mutagenised population (Kakutani et al., 1995) and transgenic plants expressing the DNA methyltransferase gene (MET1) in antisense orientation (Finnegan and Dennis, 1993; Finnegan et al., 1996). FWA (FLOWERING LOCUS WA) was isolated by a map-based approach and the DNA sequences of the wild type and the mutant alleles were identical. FWA is expressed in mutant tissues but silenced in wild type plants which is related with extensive methylation of two direct repeat sequences in the 5' region of the gene. Epigenetic fwa alleles remain stable and do not remethylate spontaneously even after several generations (Kakutani et al., 1997, Soppe et al., 2000).
In *Linaria vulgaris* methylation and silencing of the cycloidea gene (*Lcyc*) caused a change in flower morphology. Naturally occurring flower variants with radial symmetry were found to have a methylated and transcriptionally silent *Lcyc* gene. In occasional revertants the degree of phenotypic penetrance was correlated with the degree of *Lcyc* methylation (Martienssen and Colot, 2001, Habu et al., 2001).

### 5.10.4 Role of SANT motif in other proteins

*DUO3* has a myb-like DNA binding domain also known as the SANT domain. Despite its homology to the Myb-DBD, the functions of SANT domain in transcriptional regulatory factors are unknown. However, the SANT motif is present in a number of chromatin remodelling enzymes (SWI, RSC), histone acetyltransferase (Ada) and histone deacetylases (CoREST, NcoR) from yeast and mammalian cells (Aasland et al., 1996). Chromatin structure is correlated with transcriptional repression or activation and the state of chromatin is influenced by a range of mechanisms including the ATP-dependent remodelling of complexes such as Swi-sn1 and histone modifications: acetylation, deacetylation, phosphorylation and methylation (Wagner, 2003; Fransz and Hans de Jong, 2002; Guofu et al., 2002).

The first histone acetyltransferase (HAT) identified was Gcn5 and was found to be a subunit of the transcriptional regulatory complex SAGA in yeast (Brownell et al., 1996). Ada2 is an essential subunit of several Gcn5 containing complexes (Sterner et al., 2002; Boyer et al., 2002). The yeast Ada2 has a SANT motif and the role of the motif was investigated. Deletion and substitution mutations within the Gcn5 and Ada2 were analysed, which revealed an apparent dual function of the SANT domain such that SANT can be divided into two linked subdomains, SANT-a and SANT-b. SANT-b is involved in specific interaction with the acetylase Gcn5 and the second part SANT-a appeared to be directly related to interactions with or recognition of chromatin (Sterner et al., 2002). The Ada2p SANT domain is essential for histone acetyltransferase activity and kinetics analysis revealed that an intact SANT domain is required for an Ada2p-dependent enhancement of histone tail binding and catalysis by Gcn5p (Boyer et al., 2002). It has also been shown in yeast that deletion of Ada2 SANT domain causes a
strong delay in chromatin remodelling and inactivation of a native promoter. The same
defect is seen in the absence of Gcn5 histone acetyltransferase activity, this suggested
that the Ada2 SANT domain controls Gcn5 catalytic activity (Barbaric et al., 2003). In
eukaryotes histone deacetylation is correlated with transcriptional inactivity. The human
histone deacetylases I (HDAC1) and II (HDAC2) are homologous proteins that catalyse
release of acetyl groups from modified N-terminal lysines of core histones. HDAC1 and
HDAC2, as well as components of HDAC3 core complexes are distinguished by the
presence of the SANT domain proteins. It was predicted that the SANT domain might
play a general role in HDAC complex assembly (Humprey et al., 2001).

SMRT (silencing mediator for retinoid and thyroid hormone receptors) and N-CoR
(nuclear receptor thyroid hormone receptors) are transcriptional repressors that exist in
core repression complexes with histone deacetylase 3 (HDAC3) (Li et al., 2000). Both
proteins contain a closely spaced pair of SANT motifs. The functions of the SANT
motifs in SMRT and N-CoR are not clear, it has been suggested that they might
contribute to protein-protein interactions required for the assembly of nuclear repressors
complexes. The pair of SANT motifs are only distantly related to one another within the
same protein (30%) but individual motif are highly conserved between SMRT and N-
CoR. The N-terminal SANT domain forms part of a deacetylase-activation domain
(DAD) that activates HDAC3. The second SANT motif functions as part of a histone
interaction domain (HID). The two SANT motifs function synergistically to increase
enzymatic activity of HDAC3 and maintain histone deacetylation. Furthermore, it was
demonstrated that the SANT-containing SMRT HID recognises and binds to the
unacetylated tail of histone H4 (Park et al., 1999, Yu et al., 2003).
Chapter Six
General Discussion
6.1 *duo pollen* mutants represent a novel class of gametophytic mutants that specifically affect generative cell division

The first genetic screens developed to identify gametophytic mutants that affect pollen divisions and pollen cell fate were morphologically based. DAPI-stained pollen grains from a fast-neutron mutagenised population (Chen and McCormick, 1996) and an EMS-mutagenised population (Park et al., 1998) were screened and this led to the identification of a wide range of gametophytic mutants. Another screen using histochemical stains has been successful in identifying pollen mutants (Johnson and McCormick, 2001). To date a few male gametophytic mutants such as *gemini pollen* (*gem*) (Park et al., 1998); *side-car pollen* (*scp*) (Chen and McCormick, 1996); *male germ unit malformed* (*gum*), *male germ unit displaced* (*mud*) (Lalanne and Twell, 2002); *emotionally fragile pollen* (*efp*), *gift-wrapped pollen* (*gwp*), *polka-dot pollen* (*pdp*), *raring-to go* (*rtg*) (Johnson and McCormick, 2001) have been isolated in *Arabidopsis* based on morphological screens. Some of the gametophytic mutants derived from the morphological screens have been described in section 1.18. In particular, the morphological screen using DAPI staining (Park et al., 1998) yielded a novel class of mutants termed the *duo pollen* mutants. The *duo pollen* mutants represent a distinct class of gametophytic mutant that specifically fails to enter or complete generative cell division. The generative cell represents the male germ line in flowering plant and generative cell division is a key event during plant sexual reproduction.

Six *duo* mutants (*duo1*- *duo6*) arising from independent lines specifically fail to undergo generative cell division and remain arrested in a bicellular condition. These mutants are distinct from other gametophytic mutants such as *madl* (male gametophytic defective) in *Arabidopsis* (Grini et al., 1999) and *gaMS1* in maize (Sari-Gorla et al., 1997) that also fail to undergo PMII. The *madl* and *gaMS1* mutants showed bicellular arrest along with other phenotypes such as reduction in pollen size or collapsed pollen and absence of the intine layer. The pleiotrophic phenotypes observed in *madl* and
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_gaMS1_ suggest that delayed vegetative cell development indirectly affects GC division. Recently, a male sterile line segregating 3:1 was isolated from soybean and in spite a well formed generative cell, PMII failed to occur, leading to degeneration of the GC (Bione et al, 2002). In contrast to these mutants, the pollen size in all six _duo_ mutants appeared as wild type and no collapsed pollen were observed by light microscopy. Electron microscopy revealed that the vegetative cytoplasm in _duo1-duo3_ contained many small vacuoles and clusters of starch granules suggesting that maturation of the vegetative cytoplasm occurred as in the wild type (section 4.3). Furthermore, the vegetative cell specific transgene _lat52-gusnia_ was correctly expressed in the vegetative cytoplasm of mutant _duo1, duo2_ and _duo3_ pollen grains and mutant GCs correctly failed to activate the VC-specific transgene. This result indicated that vegetative cell identity is unaltered. Moreover, the _duo1, duo2_ and _duo3_ mutants germinated to form pollen tubes as efficiently as the wild type pollen grains. These results indicate that _duo_ pollen mutants specifically affect generative cell division and do not affect the development or functions of the vegetative cell. Furthermore, _duo_ mutants do not affect the sporophyte development in the heterozygous condition.

**6.2 _duo_ mutations are gametophytic and male-specific**

Genetic analysis of the _duo_ mutations (section 3.2) revealed the gametophytic nature of mutations. Expression of approximately 50% mutant pollen in heterozygous _duo1-duo3_ mutants indicated a gametophytic role of the _duo_ mutations. Genetic analysis defined _duo1, duo2, duo3_ and _duo4_ as fully penetrant gametophytic mutations whereas _duo5_ and _duo6_ were incompletely penetrant gametophytic mutations. Furthermore, tetrad analysis of _duo3_ and _duo6_ confirmed the gametophytic nature of _duo3_ and _duo6_ (section 3.3).

The finding that in _duo1- duo6_ the proportion of mutants in the F$_2$ (self) progeny was approximately 50%, or less than the expected 75%, suggested reduced gametophytic transmission for each _duo_ mutations. Analysis of genetic transmission by reciprocal crosses revealed impaired transmission through the male gametes in _duo1-duo6_ (section 3.4). Genetic transmission through the female was normal in all six _duo_ mutants. This
indicated that the \textit{duo} mutation specifically affected the development of the male gametophyte and did not compromise the development or functions of the megagametophyte.

The map positions were determined for all the six \textit{duo} mutants. It was found that \textit{duo1-6} represent different genes as they all mapped to different chromosomal locations (section 3.6.3). The chromosomal location of \textit{duo5} was not determined and it did not seem to be allelic to any of the other \textit{duo} mutants. This led to the conclusion that at least six different genetic loci control the unique division that culminates into sperm cells formation in \textit{Arabidopsis}. As these mutants represented different genes and not multiple alleles it was predicted that the screen has not reached saturation to isolate mutants that fail to enter or complete pollen mitosis II. Although disrupting a duplicated gene can result in a phenotype, an increased functional redundancy would reduce the likelihood of a phenotype overall. It was estimated that 10\% of the \textit{Arabidopsis} genes (approximately 3,000) should give a loss-of-function phenotype (Meinke et al., 2003).

\textbf{6.3 \textit{duo1}, \textit{duo2} and \textit{duo3} affect pollen mitosis II}

Developmental analysis revealed that \textit{duo1}, \textit{duo2} and \textit{duo3} fail at tricellular stage (section 4.6). In all the three \textit{duo} mutants developmental programs associated with the formation of the generative cell is coordinated correctly as in the wild type. In \textit{duo1}, \textit{duo2} and \textit{duo3} asymmetric division at PMI occurs normally and the earliest defect is only seen at PMII. Differentiation of the generative cell depends on division asymmetry at PMI, if the division at PMI is made symmetric or eliminated, the resulting cells either differentiate as vegetative cells (Eady et al., 1995) or can even be induced to develop directly into haploid embryos without fertilisation (Zaki and Dickinson, 1991). In the male meiotic mutant \textit{tetraspore (tes)}, following failure of the male meiotic cytokinesis all four microspore nuclei remain within the same cytoplasm. In the \textit{tes} mutants some nuclei complete their developmental programmes to form the functional vegetative nuclei and sperm cells, potentially the coenocytic \textit{tes} pollen grains have multiple polarities (Speilman et al., 1997). In the \textit{duo pollen} mutants asymmetric division occurred normally and presumably the partitioning of signals were also normal. These
data suggest that along with the polarised signals a more complex mechanism exist to trigger the generative cell division. The ability of duo pollen mutants to orchestrate microspore polarity and undergo the first haploid division successfully, but fail to enter or complete the second haploid division suggests that the generative cell division is presumably controlled by a different set of gene and by different mechanisms. Different sets of genes might be controlling PMI and PMII and gene redundancy can also explain the normal mitotic division at PMI in the duo mutants.

6.4 Generative cell cycle progression in duo mutants

Valuable insights into generative cell division were gained by studying mitosis in the duo mutants (sections 4.7 and 4.8). Pollen grains undergoing pollen mitosis II were scored from the bud stages -5 and -4 in both wild type and the duo mutants and the mean mitotic indices were determined. The mean mitotic index in duo1 was higher than that observed in the wild type. Moreover, duo1 showed an elevated frequency of prophase and prometaphase figures and low frequency of anaphase figures. Therefore mutant pollen grains in duo1 entered mitosis and spent longer at prophase and prometaphase but failed to undergo chromatids separation. At mature stage duo1 mutant pollen grains did not exhibit prometaphase figures but the chromatin appeared distinctly condensed and conglomerated. In alfafa cultured cells treatment with a protein phosphatase inhibitor leads to hypercondensation of late prophase chromosomes that cannot enter metaphase (Ayaydin et al, 2000). Based on the phenotype observed in duo1 it was proposed that the generative nuclei in duo1 might undergo premature condensation and proceeded to prometaphase of the M phase.

The entry and progression through mitosis is a complex process and in eukaryotic cells the initiation of mitosis is controlled by complex phosphorylation cascade that culminates in the activation of mitosis promoting factor (MPF). MPF consists of cyclin dependent kinases (CDK)(Cdc2 in yeast) and a B-type cyclin regulatory subunit. In yeast a single CDK is responsible for initiating all cell cycle transitions, whereas in higher eukaryotes the CDKs have evolved into gene families. Plants have two different
classes of CDKs that control both entry and progression into mitosis (Criqui and Genschik, 2002). CDKs must associate with cyclins that are key regulatory proteins.

In *Arabidopsis*, genome-wide analysis of the cyclin family revealed at least ten A-type cyclin and nine B-type cyclins (Vandepoele et al., 2002). In *Arabidopsis*, ectopic expression of cyclin B1;1 was found to accelerate root cell proliferation (Doerner et al., 1996). Ectopic expression of cyclinB1;2 but not cyclin B1;1 in trichomes reduced the number of endoreduplication rounds and promoted nuclear division and cytokinesis, resulting in the formation of multicellular trichomes (Schnittger et al., 2002). Myb-like proteins that interact specifically with the M-specific activator (MSA) *cis-acting* elements have been characterised in B-type cyclin promoters (Ito et al., 2001). Besides activation by cyclins, phosphorylation events regulated CDK activity. The phosphorylation of the conserved threonine residue in the T-loop of the CDK by CDK-activating kinases (CAK) is an important step in CDK activation (Stals and Inze, 2001).

In fission yeast MPF activation focuses on the phosphorylation state of the Tyr15 residue of cdc2, during interphase MPF is kept active through Tyr15 phosphorylation of Cdc2 and upon entry into mitosis MPF is activated by dephosphorylation of the residue. The onset of mitosis is triggered by the simultaneous activation of the Tyr15 phosphatase Cdc25 and the inactivation of the Tyr15 kinase Wee1. Cdc2-cyclinB is capable of activating and repressing the activity of Cdc25 and Wee1 respectively and Cdc2 activation depends in part on a positive feedback loop (Morgan, 1995; Lew and Kombluth, 1996; Ohi and Gould, 1999). In *Drosophila*, polo kinase has been identified as an activator of Cdc25 (Nigg, 1998).

Regulation of the cell cycle in plants is controlled primarily by the interactions of CDK and cyclins. It was speculated that in *duo* mutants the key regulatory factors controlling progression into mitosis might be impaired or factors modulating the activity of the CDKs and cyclins are disrupted in the *duo* mutants. However, cell cycle patterns associated with the male gametes formation within the pollen grains and the pollen tubes is remarkably diverse and it was predicted that the cyclins expressed in the male gametic cells would be highly variable among taxa (Friedman, 1999). In flowering
plants two patterns of the timing of generative cell division with respect to pollen release have recognised (Brewbaker, 1967). In 70% of plant species mature pollen are bicellular and pollen mitosis II occurs in the pollen tubes. This pattern of plesiomorphic and is found in all extant nonflowering seed plants. In contrast, 30% of angiosperm species mature pollen is tricellular at anthesis. Five patterns of sperm and male gametophyte development (section 1.14) has been recognised based on the relationships between the relative timing of sperm formation (pollen grain or pollen tube) and the timing and expression of S phase in the sperm cells.

6.5 S phase progression in the male gametic cells

Fundamental insights into the cell cycle pattern followed by the male gametic cells in wild type and duo mutants were established (section 4.9). The relation between generative cell and sperm cell ontogeny and the cell cycle was examined in Arabidopsis. A routine procedure was developed to measure the nuclear DNA content that is based on relative fluorescence emitted after DAPI-staining.

Incipient generative nuclei are appressed to the pollen wall and produce a DNA content of 1C and the DNA content increased to 1.04C soon after detachment from the pollen wall. When the generative nuclei are engulfed within the vegetative nuclei, the fluorescence value corresponded to 1.15C DNA content and reached 1.95C just before pollen mitosis II. Incipient sperm cells produce a DNA content of 1C during anaphase and telophase and the DNA content increased progressively in the subsequent stages of pollen development, such that at anthesis the sperm nuclei produced fluorescence value that corresponded to 1.20C. Earlier work has shown that the sperm rapidly enters S phase of the cell after inception and sperm cell G1 is very short or non-existent (Friedman, 1999). The above data indicate that the generative cell starts DNA synthesis soon after its formation similar to the sperm cells.

Collectively, these data suggest that the male germ line development follows a S-M cycle and the gap phase (G1) is essentially non-existent. Animal cells in early embryogenesis undergo rapid cell cycles that consist only of alternating S and M
phases. In these cells CDK controls the switch between S and M phases principally by periodic synthesis and degradation of cyclin B that is the positive regulator of CDK activity (Correllou et al., 2000). However, in other plant species DNA replication in the generative nuclei does not occur soon after formation. In newly formed generative nucleus of *Scilla peruviana* L. DNA replication in the generative cell occurs at an intermediate stage of maturation (Gonzalez-Melendi et al., 2000).

The cell cycle progression of the generative cells in *duo1*, *duo2* and *duo3* were monitored. It was found that in *duo1*, *duo2* and *duo3* the cell cycle progression of the generative nuclei before PMII were similar to wild type. In *duo1*, the generative nuclei produced 1.98C DNA content just before PMII. In *duo2* and *duo3* generative nuclei produced 2C and 1.98C DNA content respectively, suggesting that DNA replication was complete at PMII. This indicates that the molecular programme associated with DNA replication is functional in *duo* mutants and generative cells can progress through a complete S phase.

In *duo1*, no further increase in DNA content was detected and at anthesis the mutant generative nuclei contains 1.99C DNA content. In marked contrast DNA content in GCs of *duo2* and *duo3* increased progressively and at anthesis reached 2.35C and 2.46C respectively. *duo2* and *duo3* rereplicate their DNA, however, *duo2* and *duo3* pollen mutants do not produce giant nuclei that could occur as a result of several rounds of endoreduplication. Potentially, DNA synthesis licensing controls are not switched off in *duo2* and *duo3*. It was predicted earlier that the male gametic cells follow a S-M cell cycle pattern in the wild type therefore there must be a rapid synthesis and degradation of cyclin B which in turn regulates CDK activity. Investigations on the controls of S phase in yeast revealed that after the completion of S phase, cyclin/CDK activity must fall to low levels and low CDK activity during G1 allows the assembly of prereplication complexes (preRCs) licensing DNA for another round of replication (Donaldson and Blow, 1999). Presumably, *duo2* and *duo3* failed to enter M phase due to absence or aberrant mitotic cyclins and the absence of mitotic cyclins triggered entry into S phase.

6.6 Molecular mapping of *DUO3*
A map-based cloning approach was used to isolate the *duo3* gene. For fine mapping of a mutation, it is necessary to analyse a large number of progeny especially if the locus falls in a chromosomal region of low recombination frequency. In case of *duo3*, approximately 821 plants were analysed and the *duo3* locus was narrowed down to a 10 kb region (section 5.4) containing two hypothetical genes (At1g64570 and At1g64580). Both genes from the *duo* locus including the intergenic region were sequenced from heterozygous mutant plants. However, no base change were detected that could cause truncation of the gene.

Proof of successful gene identification and cloning usually requires complementation of the mutant phenotype by transformation with a wild type allele. To accelerate positional cloning in plants plant competent vectors such as λ-phage vector λT12 have been developed for construction of genomic libraries with inserts of 5-25 kb that are used for genetic complementation of mutants (Fuse et al., 1995). Recently, a TAC vector, pYLTAC7, was developed to decrease the difficulty in positional cloning. This vector is suitable for stable maintenance of large genomic DNA fragments in both *E. coli* and *A. tumefaciens* and is competent for transfer of insert DNA into a plant genome by *Agrobacterium*-mediated transformation (Liu et al., 1999). A different strategy was used to complement *DUO3* (section 5.6). The ORFs of the genes At1g64570 and At1g64580 including 1 kb upstream and 1 kb downstream the genes were cloned into pPZP221 and used to transform *Arabidopsis*. Revertants were obtained from the construct pPZSAT, which showed a reduction in the number of mutant pollen. Male transmission was restored up to 55% in these revertants (section 5.7). This data led to the conclusion that the gene At1g64570 represents *DUO3*.

Despite the evidence that *duo3* can be complemented with the gene At1g64570, sequencing of *DUO3* coding sequence from *duo3* revealed no nucleic acid sequence differences from the wild type. These data strongly suggest that *DUO3* is an epiallele. A well-documented example for heritable changes in the gene expression that are not related to differences in the underlying DNA sequences is the SUPERMAN gene and the *clark kent* (clk) alleles of *Arabidopsis*. Sequencing of the SUP coding region from
In *Arabidopsis*, MET1 gene, a homolog of the mammalian Dnmt1 is required during gametogenesis and depletion of MET1 results in immense epigenetics diversification of gametes. Complete depletion of the MET1 activity resulted in gametophytic effects (Saze et al., 2003).

### 6.7 DUO3 has a SANT domain

Database searches revealed that the predicted DUO3 protein has a myb-like DNA binding domain also known as the SANT domain (section 5.9). The *MYB* gene was first identified as a *v-Myb* oncogene derived from the avian myeloblastosis virus (Klempnauer et al., 1982). Other genes related to the *v-Myb* have been identified in human, plants, fungi and insects. The *Myb* genes share a MYB DNA-binding domain and this domain comprises of up to three imperfect repeats each forming a helix-turn-helix structure of about 53 amino acids. In each Myb repeat three highly conserved tryptophan residues are evenly spaced (Lipstick, 1996). In plants, MYB proteins are categorised into three subfamilies based on the number of repeats in the MYB domain (Jin and Martin, 1999). In *Arabidopsis* the MYB families of transcription factors have been strongly amplified and about 131 members belong to the MYB-R2R3 class (two MYB repeats) and five proteins to the MYB-R1R2R3 (three MYB repeats). In addition, *Arabidopsis* contains a more divergent MYB domain, which is characterised by a single repeat. They are referred as Myb-like proteins (Riechmann et al., 2000). It has been shown that MYB-like proteins with single repeat act as transcriptional activators (Baranowskij et al., 1994). CCA1 (Circardian Clock Associated1) and LHY (Late Elongated Hypocotyl) have one-repeat MYB domain and are involved in signal
transduction and regulation of the circadian clock (Schaffer et al., 1998; Wang et al., 1997). The role of several R2R3-type MYB transcription factors have been characterised and it mainly regulate plant-specific processes (Stracke et al., 2001). The AtMYB26 is a R2R3-type transcription factor and has been shown to be required for male fertility in *Arabidopsis* (Steiner-Lange et al., 2003). Two cloned genes required for male-specific development that encode transcription factors are *NOZZLE* and *MSI*. *NOZZLE* encodes a protein that belongs to the MADS-domain protein family (Schiefthaler et al., 1999) and *MSI* belongs to the PHD finger class (Wilson et al., 2001).

*DUO3* has a single SANT motif. For proteins with single SANT motif, the role is not clear. However, SWI-SNF and TFIIB contain single SANT domain and have considerable affinity for DNA. The role of the SANT motif, especially in plants is unknown but potential transcriptional and chromatin related functions has been put forward from studies in yeast and human (described in section 5.10.4). The SANT domain is found within the subunits of the ATP-dependent chromatin remodelling enzymes such as yeast Swi3p, Rsc8p, human BAF155/170 and Drosophila ISWI (Aasland et al., 1996). It is also present in human histone acetyltransferase (Ada2p), in histone deacetylase (HDAC3) and in nuclear receptor corepressors such as SMRT (Sterner et al., 2002, Park et al., 1999, Humphrey et al., 2001).

Several models have been proposed for the involvement of SANT motif in transcriptional regulation. The SANT motif might mediate DNA binding like the Myb proteins or play a role in protein-protein interactions (Aasland et al., 1996). In yeast the functional importance of Ada2 SANT region was investigated by creating deletion and substitution mutants in Ada2 and Gnc5. Based on the results the role of the Ada’s SANT domain in chromatin modification have been speculated. SANT motif may interact directly with histones to stabilise the Gcn5’s substrate binding and allowing more efficient acetylation. Another possible function of the SANT motif is that SANT confers specificity for nucleosomes as a substrate, and interacts preferentially with nucleosomal structures rather than free histones (Sterner et al., 2001). Ada2 is a component of the Gcn5p HAT complexes and the involvement of Ada2p SANT was
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investigated. Based on the result a model was established in which the SANT domain might interact with Gcn5p and thereby alter the active site. The SANT motif may interact directly with the histone tail and modify it in the proper conformation for efficient binding and catalysis of Gcn5p (Boyer et al., 2002). It has been shown that the two SANT motifs in the nuclear receptor corepressors, SMRT and NcoR, play a critical role in the optimal delivery of histone substrates to chromatin-modifying enzymes (Yu et al., 2003).

6.8 Hypotheses for the mode of action of DUO3

Several alternative hypotheses are proposed for the potential function of DUO3 during generative cell division based on the presence of the SANT motif. Three models are proposed,

1) in the first model DUO3 might bind directly to DNA and promote transcription,
2) in the second model DUO3 acts as or in association with a chromatin-remodelling enzymes, making the DNA in the generative cell more accessible for transcription.
3) thirdly, DUO3 might bind to a transcriptional activator (eg. HAT complexes) and promote transcription.

In the first model, DUO3 might bind to DNA by virtue of its SANT domain just like the Myb domain and promote transcription. Potentially, DUO3 could recognise specific DNA sequences and binds directly to DNA. The DNA binding activity of the SANT domain has been proposed in many cases (Aasland et al., 1996; Sterner et al., 2002).

In the second hypothesis, a transcription factor binds to nucleosomal DNA and recruits a chromatin remodelling enzyme. It has been suggested that the SANT domain might be adapted for protein-protein interactions (Aasland et al., 1996). Therefore DUO3 might interact with other proteins and function as a remodelling enzyme in the generative cell. It has been shown that SANT domain is present in chromatin remodelling enzymes such as SWI/SNF and RSC and an intact SANT motif is essential for the functioning of these enzymes (Boyer et al., 2002).
One distinct morphological feature of the generative cell is the highly condensed chromatin. The condensed state of the generative cell is usually associated with low transcriptional activity (McCormick, 1993; Tanaka, 1997; Twell et al., 1998). In general histones associated with transcriptionally inactive DNA are hypoacetylated and are rendered active by the addition of acetyl groups by histone acetyltransferases (HATs). HATs do not function in isolation but usually form part in a variety of large subunit complexes such as TFIID and the SAGA complex (Goodrich and Tweedie, 2002). The best-characterised HAT in yeast is the GCN complex that contains Ada2p proteins (section 5.10.4). The Ada2p contains a SANT domain that is essential for HAT activity and functions as a histone H4 tail-presenting module (Boyer et al., 2002). DUO3 might have similar functions as Ada2 that is to promote HAT activity. DUO3 could mediate a physical or functional interaction with the histone H4 N-terminal domain by virtue of its SANT domain and promote specific histone acetylation at gene level in the generative cell.

6.9 Conclusions and perspective

Isolation of the *duo pollen* mutants (*duo1-duo6*) provided evidence of haploid expressed genes that specifically control generative cell division. Mapping data revealed that the *duo1-duo6* represent different genes and at least six different genetic loci control the unique division that leads to sperm cells formation in *Arabidopsis*. This study has also uncovered new insights into understanding the basic programs associated with the cell cycle progression of the generative and sperm cell. Moreover, the isolation of the *DUO3* gene has been an important step towards identifying gene involved during gametophytic phase.

The *DUO3* is a unique gene in *Arabidopsis* and it will be a fascinating task to exploit this gene that is responsible for the generative cell division. The expression patterns of *DUO3* during development of the male gametophyte, particularly in the generative cell, can be monitored by *in-situ* hybridisation. In relation to the function of DUO3 protein further investigations are required. *DUO3* gene could be expressed as a purification-
tagged protein in *E.coli* using a his-tagged pET vector and purified DUO3 protein can be used to raise antibodies for immunolocalisation and immunoprecipitation study. An insight into DUO3 function can be potentiated by under or misexpressing DUO3 protein in sporophytic cells. First, sense and antisense *DUO3* RNA behind a strong constitutive promoter, cauliflower mosaic virus 35S promoter (CaMV35S), may be introduced into *Arabidopsis*. However, CaMV 35S is not truly constitutive and if constitutive DUO3 downregulation or ectopic expression causes lethality, a targeted antisense/misexpression strategy could be used using GAL4 enhancer trap lines (Berger et al., 1998).

Moreover, protein-protein interactions of DUO3 must be identified. The SANT domain is a novel motif present in the DUO3 and the function of this domain in plants is unknown. A challenging task would be to understand the role of this motif and to find out the interacting partners of DUO3. Co-immunoprecipitation and/or affinity chromatography can be used to assess *in vitro* interactions and *in vivo* using the yeast two-hybrid system. DUO3 protein as a bait could be used to remove protein partners from a pollen library in *Arabidopsis*.

In the post-genomic era the genome wide analysis of gene expression has a profound effect on the discovery of pollen gene expression and functions. Microarray technology has demonstrated the diversity of the haploid genome in *Arabidopsis thaliana* (Honys and Twell, 2003, Becker et al., 2003). Moreover, significant advances have been in the isolation of generative and sperm cells. The c-DNA library prepared from partially purified *Nicotiana tabacum* sperm and maize sperm library has an important impact on the discovery of sperm gene expression (Xu et al., 2002, Engel et al., 2003). Highly pure sperm of *Zea mays* was obtained by fluorescence-activated cell sorted (FACS) and a high-quality cDNA library was constructed. Sequencing of over 1100 cDNA from the amplified library revealed that sperm have a diverse complement of mRNAs. Several transcripts encoded hypothetical proteins and proteins of unknown functions (Engel et al., 2003). Detailed molecular information can be uncovered from the sperm cell library to understand the development of the male gametophyte.
Appendix
## Appendix

### Mapping data of duo3

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#### Notes:
- The mapping data represents the relationships between the original and reduced datasets.
- The matching process identifies similar patterns or correlations between the data sets.
- The target column signifies the outcome or result of the mapping process.
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**Note:** The table appears to be a grid with rows and columns, possibly indicating a form or a list of items. Without additional context, it's challenging to provide a clear interpretation. It seems to involve some sort of categorization or listing, possibly related to a form with fields labeled as 'W T C'.
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Bibliography


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