THE IDENTIFICATION OF GENES CONTROLLING
STAMEN TISSUE SPECIFICATION AND DIFFERENTIATION
IN ARABIDOPSIS THALIANA

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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October 1997
Dedicated to my father Mr. G.B.A. Sørensen,
in recognition of his achievements and aspirations.
Acknowledgements

Firstly, I would like to thank everyone in Botany for the all their help and the great atmosphere in the department. Thank-you to Dr. François Guerineau for his help and advice throughout my laboratory work. I would like to show my special appreciation to Dr. Rod Scott for his guidance and proof reading of this manuscript - all the best to you and your family in your new position at Bath. Thank-you also to Dr. Bernard Mulligan for the use of the γ-irradiation source at Nottingham University. I am also grateful to Simon Byrne, for the hours spent helping me with the in situ hybridisation and for the last minute corrections. Thanks Simon - all the best. Thanks also to Stefan and Evie in the electron microscopy laboratory for their help and advice. I am also grateful to the BBSRC for funding this project.

This thesis and my sanity remain intact solely due to the perpetual moral support from all my family: Maria, Caroline and Steve, Peter and Michaela, Jane and Vince and especially my nieces and nephews, Tom, Katie, Charlie, Kevin, Suzanne and Jack - cheers folks. In the same token, a big thanks to Pauline Bablak for managing my stress levels - Nice one Pol. I'm also grateful to Penny and Richard for the use of their caravan over the summer - Thank-you.

Just one last thanks to everyone who has provided me with accommodation and support while I completed the last few weeks of writing, especially my sister Jane.
The identification of genes controlling stamen tissue specification and differentiation in *A. thaliana*. A. M. Sorensen.

This thesis describes the generation and screening of an *A. thaliana* M2 population for mutant lines affected in stamen development. The strategy utilised, was to mutagenise transgenic *A. thaliana* seed, homozygous for either the A6 or A9 promoter linked to the *GUS* gene, using γ-irradiation. The temporal expression patterns of the A9 and A6 promoters were evaluated for their use as genetic markers to delimit windows along the stamen differentiation pathway, into which subsequently isolated mutants could be placed. The *TMS2* gene was also evaluated for its use as a visual reporter gene to aid in the isolation of mutant lines. The resulting M2 population was screened to identify mutants of the stamen differentiation pathway, by assaying for the absence or reduction of GUS activity in the floral buds. Four non-allelic male sterile mutants were identified and characterised, namely *gne1, gne2, gne4* and *gne7*. Observations of anther histology show the abnormal differentiation of the tapetum and middle layer cells in *gne1* and *gne4* and the abnormal specification of these two tissue types in *gne2*. The fourth mutant line, *gne7*, shows asynchronous microspore development. Genetic map positions were ascertained for *gne1, gne2* and *gne7* using the *A. thaliana* W100F multimarker line and CAPS analysis. Additionally, CAPS analysis revealed that the *PHYC* gene was deleted in the *gne7* mutant. This thesis also examines the sense and antisense expression of the *AG* gene under the control of the A6 and A9 promoter, as well as the analysis of the A9 promoter by 5' deletion analysis.
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Chapter 1
Introduction
Introduction

_A. thaliana_ is a dicotyledenous plant species that belongs to the mustard family (cruciferae). Initial interest in _A. thaliana_ as a research tool was realised through its favourable life cycle and low chromosome number (Laibach in 1943), and the subsequent development of applicable molecular biology techniques, has since elevated this small plant to the status of a model species. _A. thaliana_ has a small and simple genome, estimated by DNA reassociation kinetics at $1 \times 10^8$ bp (Meyerowitz and Pruitt, 1985), which contains a low amount of repetitive DNA (Leutwiler et al., 1984). The genome organisation is far simpler than in other higher plants, and proteins encoded by single genes or small gene families in _A. thaliana_ are encoded by multiple genes or large gene families in other species (Meyerowitz and Pruitt, 1985). _A. thaliana_ can be easily grown, under artificial lighting, in a mixture of soil and sand and has a generation time of approximately 5 weeks. These diploid hermaphrodite plants are small, enabling dozens to be grown per pot, and each individual can produce over 10 000 seeds. _A. thaliana_ can be grown on sterile selective media and various methods of transformation exist using _A. tumefaciens_, such as leaf and cotyledon transformation (Schmidt and Willmitzer, 1988), vacuum infiltration (Bechtold et al., 1993), seed transformation (Feldmann and Marks, 1987) and root transformation (Valvekens et al., 1988).

Many mutants have been isolated (Estelle and Somerville, 1986) and seed stocks can be obtained from central seed banks. Mutagenesis of seed stocks by genetic, chemical or irradiation techniques can be used to generate new mutations. The availability of seed stocks containing visible markers, e.g. W100F (Koornneef et al., 1987), or genetic markers, e.g. the RI lines (Lister and Dean, 1993), and the existence of polymorphisms utilised in RFLP (Chang et al., 1988; Nam et al., 1989) and CAPS mapping (Konieczny and Ausubel, 1993) enable new mutations to be mapped onto the existing combined physical and genetic map (Jarvis et al., 1994). Once accurately mapped, clones from genomic libraries, in the area of interest, enable the isolation of the mutated gene in question, by chromosome walking and complementation. Mutants generated by DNA insertion techniques, such as transposon tagging (Aarts et al., 1993), or T-DNA insertional mutagenesis (Feldmann, 1991), enable the mutated genes to be isolated by inverse polymerase chain reaction (IPCR) (Ochman et al., 1988). If the mutant line generated contains a deletion, as is often caused by irradiation exposure, genomic subtraction cloning (Straus and Ausubel, 1990), may be used to isolate the gene. These molecular
techniques required to evaluate and isolate mutated genes, secure *A. thaliana* as an ideal species for the study of plant development. The ability to cross hybridise genes cloned from *A. thaliana* with a wide variety of both monocotyledenous and dicotyledenous plant species also enables genes of interest, cloned from *A. thaliana*, to be used as probes to isolate homologous genes from plants of economic value.

### 1.1 Flower development in *A. thaliana*

In *A. thaliana*, flowers develop from floral meristems, a group of primordial cells that divide and differentiate into four different types of floral organs, the sepals, petals, stamens and carpels. In wild type *A. thaliana*, each flower consists of four concentric whorls, comprising an outermost whorl of four sepals, leading to four petals, six stamens and a gynoecium of two fused carpels at the centre. The genetic study of floral mutants of *A. thaliana* has identified some of the genes concerned in the floral developmental pathway, and given an insight into the signalling mechanisms involved in this complex pathway of cell division, growth and differentiation (Okada and Shimura, 1994; Weigel and Meyerowitz, 1994). Initial expression of the floral meristem identity genes, at the shoot apex, triggers the transition of the vegetative meristem to an inflorescence meristem from which floral meristems are produced. Cadastral genes then act within the floral meristems to spatially regulate the floral homeotic genes, whose subsequent expression patterns, specify floral organ position and identity.

#### 1.1.1 Floral homeotic genes

Studies on floral homeotic mutants has lead to the proposal of a model, known as the ABC model (reviewed in Weigel and Meyerowitz, 1994), which predicts floral organ identity in *Arabidopsis*. The model defines three classes of floral homeotic genes. Class A includes *APETALA1 (AP1)* and *APETALA2 (AP2)*, which are expressed in the first and second whorls (Bowman et al., 1989, 1991b, 1993; Kunst et al., 1989; Irish & Sussex 1990). Class B includes *APETALA3 (AP3)* and *PISTILLATA (PI)*, which are expressed in the second and third whorls (Bowman et al., 1989, 1991b; Hill & Lord 1989). Class C includes *AGAMOUS (AG)*, which is expressed in the third and fourth whorls (Bowman et al., 1989, 1991a, 1991b; Drews et al., 1991). This model allows unique combinations of gene expression to specify organ identity. Such that class A activity alone specifies
sepals in the first whorl. Class A and B activity specifies petals in the second whorl.
Class B and C activity specifies stamens in the third whorl. Class C activity alone
specifies carpels in the fourth whorl.

Mutations in any of these three classes of genes, leads to the homeotic
conversion of the floral organs in the two whorls, where the gene is expressed in
wild type flowers. Floral homeotic conversions can be predicted, by the fact that
class A and class C genes act antagonistically, such that in the absence of class A
gene expression, class C gene activity extend to the first and second whorls, and in
the absence of class C gene expression, class A extends to the third and fourth
whorls (Bowman et al., 1991b). AP2 and AG can therefore be considered as
cadastral genes because they define the A and C boundary, by negatively
regulating each other and confine class A function to whorls one and two, and class
C to whorls three and four.

The floral homeotic genes, AP1, AP2, AP3, PI and AG have been cloned
(Yanofsky et al., 1990; Jack et al., 1992; Mandel et al., 1992b; Goto and
Meyerowitz, 1994; Jofuku et al., 1994), and four out of the five - AP1, AP3, PI and
AG encode putative transcription factors, with a MADS domain (Schwarz-Sommer
et al., 1990), which functions in DNA binding and protein dimerisation (Yanofsky et
al., 1990; Jack et al., 1992; Mandel et al., 1992b; Goto & Meyerowitz, 1994). The
fifth, AP2 encodes a novel, putatively nuclear protein with two 68 amino acid
repeat motifs (Jofuku et al., 1994).

In situ hybridisation experiments show RNA expression patterns of the floral
homeotic genes generally appears consistent with their proposed domains of
function. However AP2 is not restricted to whorls one and two as predicted, but is
detected throughout the floral meristem as well as in vegetative tissue (Jofuku et al.,
1994). AP3 and PI are largely expressed in whorls two and three, although PI is
initially expressed in whorls two, three and four (Jack et al., 1992, 1994; Goto &
Meyerowitz, 1994). AP1 is expressed in whorls 1 and 2 in stage 3 flowers and
older, but is initially expressed in entire flower primordium (Gustafson-Brown et al.,
1994).

1.1.2 Floral meristem identity genes

Initial expression of AP1 in the entire flower primordium is consistent with
the role of AP1 as a floral meristem identity gene (Irish and Sussex, 1990; Weigel
et al., 1992; Bowman et al., 1993; Schultz and Haughn, 1993; Shannon and
Meeks-Wagner, 1993; Mandel and Yanofsky, 1995). Other floral meristem identity
genes include LEAFY (LFY), UNUSUAL FLORAL ORGANS (UFO), CAULIFLOWER
(CAL), AP2, TERMINAL FLOWER (TFL) and TOUSLED (TSL). Mutations in the
floral meristem identity genes and their resulting phenotypes, both in single and
double mutants, reveal close interactions between the floral meristem identity
genes and the floral homeotic genes (Shannon and Meeks-Wagner, 1991; Levin
Mutations in the LFY gene cause a decrease in the class B gene expression, but
not classes A and C. In the double mutant ap1 Ify, an even greater decrease in
class B and C genes is seen (Weigel & Meyerowitz, 1993). AP1 acts downstream
of LFY to specify floral meristem identity, correlated by the fact that an ap1
background attenuates the 35S::LEAFY phenotype (the ectopic expression of
35S::LEAFY is capable of converting inflorescence shoot meristems into floral
meristems) (Weigel and Nilsson, 1995). Thus, AP1 and LFY can be considered
positive regulators of the floral organ identity genes. Using anti-LFY antiserum,ufo
mutants showed no expression of LFY gene after stage 6 of flower development,
and anti-AP3 and anti-PI antiserum also showed reduced levels of expression of
AP3 and PI after stage 6 development (Levin and Meyerowitz, 1995). ufo Ify
double mutants resembled Ify mutants showing the close interaction of UFO and
LFY, and ufo ap1 double mutants showed conversion of flowers to partial
inflorescences, showing close link between UFO and AP1. Therefore LFY is
epistatic to and downstream of UFO. As well as being involved in floral meristem
identity and pattern formation of the floral organs, UFO also shows a role in
determinacy along with AG; ufo ag double mutants show fasciation of the floral
meristem (Levin and Meyerowitz, 1995).

1.1.3 Cadastral genes

Once the floral meristem identity genes have specified a floral fate for the
shoot meristem, cadastral genes set the boundaries of floral homeotic gene
function. Cadastral genes identified to date include AG, AP2, SUPERMAN (SUP)
and LEUNIG (LUG). SUP is required to set the border between the third and fourth
whorls. sup mutants contain extra stamens in the third whorl and reduced
carpelloid tissue in the forth whorl (Schultz et al., 1991; Bowman et al., 1992).
Cloning of the SUP gene and subsequent in situ hybridisation studies revealed
that SUP expression is limited to a subset of AP3 expressing cells that form a
border between the third whorl stamens and the fourth whorl carpels (Sakai et al., 1995). *lug* mutations cause the ectopic expression of class B and C floral homeotic genes. In *lug* mutants, sepals are frequently petaloid, staminoid or carpelloid. Petals are often staminoid and reduced in number, stamens are also reduced in number and carpel number varies from 1.5 - 4. *LUG* is a class A cadastral gene, whose main role is to negatively regulate *AG*, enhanced by *AP2* expression (which plays a more major role in *AG* repression) (Lui and Meyerowitz, 1995).

1.2 Flower differentiation and androgenesis

Once the floral meristem has been established, flower development including androgenesis and microgametogenesis follows a programmed sequence of events, which in the case of the anther leads to the production of mature fertile pollen. The developmental changes occurring along the stamen differentiation pathway can be broken down into progressive stages, recognised by flower or anther morphology, or landmark events occurring within the microsporangia of the anthers.

1.2.1 Floral differentiation

Detailed scanning electron microscopy studies, on developing wild type buds of *A. thaliana*, has revealed the sequence of events in floral differentiation. Floral buds are initiated as a protrusion of cells from the side of the apical floral meristem. The sepal primordia are the first to appear from this buttress of cells, with the abaxial first, then the adaxial followed by the two lateral sepals. These primordia arise as ridges which lengthen and curve inward, beginning to overlie the dome shaped primordium. Pedicel elongation, initiated when the sepal primordium appear, continues and the growth of the floral bud becomes more vertical.

The stamen primordia become initiated after the sepal primordia have started to grow over the floral bud. The primordia of the four medial (longer, inner) stamens emerge first, appearing as wide outgrowths on the central dome of cells. At this stage the four petal primordia, which arise between the sepals are barely visible. Later in this stage the two lateral (shorter, lower) stamen primordia appear. As anther development proceeds the bud becomes fully enclosed by the sepals and the stamen primordia bulge out, medial first followed by the lateral, to become distinct from the central dome. The petal primordia increase in size and a central
The rim of cells grows upward to produce an oval hollow tube which will become the gynoecium. The stamens become stalked towards their bases and the vertical growth of the slotted gynoecium tube keeps pace with medial stamens. The petal primordia have now grown hemispherical in shape. The locules of the anthers become visible as convex protrusions on their inner side and petal growth accelerates and the petal primordia become stalked. Rapid growth and elongation of the floral organs takes place, and the sepals still surrounding them grow accordingly to accommodate the change in pace. The petals reach the top of short stamens and the gynoecium develops stigmatic papillae. The stamens and gynoecium elongate in concert, until the anthers have almost reached their mature length and the upper part of gynoecium differentiates into the style. The sepals finally open revealing a fully developed flower. Petal and stamen elongation continues until the stigma becomes receptive and anthesis occurs (Smyth et al., 1990; Goldberg, 1988).

1.2.2 Anther histodifferentiation and microgametogenesis

*Arabidopsis thaliana* flowers contain six stamens, and each stamen is composed of an anther and a filament. The anther is a complex floral organ system, composed of specialised tissue types, which is responsible for the formation of the male gametes or pollen grains and the filament which provides anchorage, support and nutrients to the developing anther. Each anther contains four microsporangia or pollen sacs, linked to each other and to the filament by connective tissue. The microsporangium wall of the anther consists of four concentrically arranged cell layers with the epidermis outermost, then the endothecium, the middle layer and the tapetum innermost (Fig. 1.1). Central to these four layers is the sporogenous tissue, comprising the pollen mother cells or meiocytes, which differentiate to form the pollen grains.

Stamen differentiation can be divided into consecutive phases of development, delimited by morphological and cytological events occurring during androgenesis. Initially the morphology of the anther is established, involving cell specification and tissue differentiation, resulting in the formation of the four microsporangia (Goldberg et al., 1993; Koltunow et al., 1990). Following the histodifferentiation of the anther, microgametogenesis occurs (Fig. 1.2), which can be divided into three phases, comprising microsporogenesis, microspore development and microspore maturation (Mascarenhas, 1989; McCormick, 1993;
Scott et al., 1991a, 1991b). This process of pollen development is accompanied by anther and filament growth, and later on specified tissue degeneration and finally dehiscence of the anther wall to the release the mature pollen grains (Goldberg et al., 1993; Koltunow et al., 1990).

Anther primordial cells are initiated from floral buds, after the sepal primordial cells have arisen (1.2.1), and comprise a mass of undifferentiated tissue which are surrounded by a discrete epidermal layer. Archesporial cells arise at the four corners of the developing anther in columns of cells, which divide by periclinal divisions to create a sub-epidermal primary parietal layer and an inner primary sporogenous layer. The primary parietal layer divides and differentiates to form three cells layers, the endothecium, the middle layer and the tapetum. The tapetum is a highly specialised glandular tissue supplying nutrients, enzymes and structural materials to the developing pollen grains (1.3.1) and the endothecium, along with another specialised but small group of cells, the stomium, are involved in dehiscence. The cells of the primary sporogenous layer develop to form the sporocytes, which initially tightly pack the anther locule and have a very angular shape.

Microsporogenesis, the differentiation of the sporocytes, involves several rounds of mitotic division to form diploid meiocytes that enter meiosis to form a tetrad of haploid microspores, separated by a callose wall. Initially the sporogenous cells undergo several rounds of mitotic division and the cells progressively enlarge, become more rounded and show a separation between their cytoplasm and their microsporocyte wall. The stimulus for the meiocytes to cease mitotic division and enter meiosis is unknown, but the onset of meiosis is simultaneous between meiocytes of different locules, anthers and even buds of a floret. The meiocytes ready to enter meiosis, then become arrested at the G1 stage of meiotic interphase by an unknown factor(s), which a matter of hours later also arrests the tapetum at this stage of development. The meiocytes are then released from this hold in synchrony and both the meiocytes and the tapetum enter DNA synthesis together (Heslop-Harrison, 1966a, 1966b).

During prophase I of meiosis, the meiocytes synthesise and secrete callose, a β-1,3 glucan, which is deposited along the plasma membrane underneath the original cellulosic wall. This thick callose deposition forms a coat covering the entire meiocyte, separating them from one another. Meiosis results in the formation of four haploid nuclei, which become separated through simultaneous cytokinesis.
in the case of *A. thaliana*, as for most dicotyledenous plants, which is accompanied by cell plate formation. During cytokinesis additional callose deposition occurs, resulting in a tetrad of haploid microspores, each surrounded by a complete callose shell. While it has been shown, in tobacco, that the premature dissolution of the callose wall causes male sterility, the meiocytes are still able to undergo meiosis and pollen abortion occurs shortly after microspore release (Worrall et al., 1992). During microsporogenesis the tapetal cells have become enlarged, vacuolate and develop a complex ultrastructure, indicating their high active metabolism during this period of development. The cells of the middle layer have become more flattened, while the epidermis and endothecium have increased proportionally in size, but remain relatively unchanged.

Almost immediately following cytokinesis in the post-meiotic meiocytes, the formation of the pollen wall begins. While the microspores are still held in the tetrad, cellulosic primexine is laid down outside the plasmalemma of the young microspore, beneath the callose coat which acts as a mould for exine deposition. Evidence for callose acting as a mould for exine deposition, is shown by transgenic tobacco microspores that lack or have greatly reduced levels of callose, and do not posses the sculpturing characteristics of WT microspores (Worrall et al., 1992). The primexine around the microspores acts as a matrix into which the exine wall polymers - protosporopollenin and sporopollenin - are deposited, synthesised from precursors present in the microspore cytoplasm; however most of the sporopollenin deposited around the microspore is derived from the tapetal cells (Knox, 1984). Once a partially formed exine coat has been laid down, the callose is dissolved from around the tetrad by the action of callase (Frankel et al., 1969), a β-1,3-glucanase secreted by the tapetum (Stieglitz and Stern, 1973). The breakdown of the callose causes the dissolution of the tetrad and microspore release. Following the release of the microspores into the locular space the tapetal cells synthesis and secrete the majority of sporopollenin required to form the outer wall, or exine, which gives the pollen a predetermined and characteristic sculptured surface (Knox, 1984). The final wall layer to be laid down by the pollen grain is an internal pecto-cellulosic wall, called the intine, which has an important role in the formation of a wall aperture through which the pollen tube grows out (Knox, 1984; Scott, 1994; Bedinger, 1992; Blackmore and Barnes, 1990; Mascarenhas, 1975).

By microspore release the anther has switched to a degeneration and dehiscence programme, the tapetum has already started to degrade and the
middle layer has become more flattened. Neither the tapetum nor the middle layer are present prior to anthesis, whereas the endothecium and the epidermis remain intact. Microspore development ends when the microspore nucleus undergoes asymmetric mitosis, followed by cytokinesis to produce a large vegetative cell and a small generative cell, resulting in a binucleate microspore.

Once the microspores have become binucleate the tissue around the stomium, the circular cell cluster, has already started to degenerate and eventually the two pollen sacs from adjacent theca merge to form a single locule. Following tapetal degeneration the endothecium cells undergo radial elongation and the formation of fibrous band thickenings also occurs along the endothecial cell walls (Chauhan, 1979). The subsequent dehydration of the endothecium and the rupturing of the anther at the stomium leads to dehiscence and the release of mature pollen grains (Keijzer, 1987).

1.3 The tapetum

The tapetum is a specialised glandular tissue that forms the innermost layer of the microsporangium wall. A single layer of tapetal cells lies beneath the middle layer cells and completely surrounds the sporogenous tissue (Fig. 1.1). In both monocotyledenous and dicotyledenous plant species during initial stamen development, the anther primordia are made up of three concentric precursor cell layers L1, L2 and L3. In most dicotyledenous plant species, including tobacco, the cell lineage of the tapetum is derived from the L2 and L3 layers (Satina and Blakeslee 1941; Esau, 1977; Koltunow, 1990). Tapetal cells on the external bulging face of the sporangium, originate from the inner secondary parietal cells, derived from the L2 primordial layer, and the resulting cells are regular in shape, and easily distinguish from surrounding cells. Tapetal cells arising on the internal sunken face of the microsporangium, usually originate from the L3 cells of the connective tissue. The divisions of these cells are less regular and in early microsporangium development it is more difficult to distinguish between tapetal and surrounding cell types, although as anther development proceeds, the tapetum becomes a physiologically homogeneous and uniform layer (Periasamy and Swamy, 1966). In A. thaliana however, the cell lineage of the microsporangium appears to follow a pattern of differentiation more typical to monocotyledenous plant species, such as that seen in Triticale (Bhandhari and Khosla, 1982; Bhandhari, 1984), where the tapetal layer is derived from a single precursor cell type, the L2 primordial layer. In
Triticale, the tapetum originates as a continuous, concentric layer around the sporogenous cells, derived solely from the inner secondary parietal tissue and is not of dual origin (Bhandhari and Khosla, 1982).

1.3.1 Role of the tapetum

The tapetum exhibits characteristic and acute morphological changes during sporogenesis, and is highly metabolically active between meiocyte meiosis and microspore release, after which time the tapetal cells gradually undergo degradation (Esau, 1977; Heslop-Harrison, 1972; Pacini, 1985). The specialisation and functions required of the tapetum during sporogenesis, is probably the reason for this cell type being responsible for producing the most abundant mRNA species, within the developing anther, indicated by tapetal-specific cDNA's being the most abundant clones to be isolated from anther cDNA libraries (Koltunow et al., 1990; Scott et al., 1991a) (1.3.2). Abnormal tapetal form or function invariably leads to male sterility (Laser and Lersten, 1972), and in higher plants the majority of male sterile lines exhibit an abnormal or persistent tapetum (Kaul, 1988). Although in some cases, tapetal lesions may be a manifestation, rather than the cause of male sterility, it does indicate the fine balance that exists between normal tapetal function and the continued differentiation of the sporogenous cells. Furthermore it has been shown that the premature and selective destruction of the tapetum leads to male sterility (Koltunow et al., 1990; Mariani et al., 1990; Paul et al., 1991; Guerineau et al., in press).

The positioning of the tapetum, enveloping the sporogenous cells, indicates that the tapetum must play a role in the supply of nutrients (Echlin, 1971), which may either simply pass through the tapetum into the locule, or be synthesised by the tapetal cells. The influence of the tapetum on the meiocytes, during meiosis is unknown, but cytoplasmic channels exist between the meiocytes and the tapetal cells up until prophase I, when the deposition of the callose wall severs these links. By the mid tetrad stage of microsporogenesis the tapetal cells have become highly metabolically active and show characteristics of secretory tissue, judged by the presence of numerous organelles, including well developed mitochondria, prominent rough and smooth endoplasmic reticulum and conspicuous plastids.

Tetrad dissolution is achieved through the enzymatic digestion of the surrounding callose coat, by the enzyme callase, synthesised by the tapetum, and secreted into the anther locule (Eschrich, 1961; Stieglitz and Stern 1973).
Following microspore release the tapetal cells then synthesise sporopollenin, a complex polymer derived from phenolics and fatty acid derivatives, which is secreted into the locule, and required to form the exine of the developing pollen grain wall (Scott, 1994). Around this time the tapetum starts to degenerate, and through this process is thought to confer compounds, required for the final stages of pollen maturation and for subsequent pollen function during pollen-stigma interactions, involved with the control of compatibility. Such compounds may include derivatives of tapetum like lipids (Heslop-Harrison et al., 1973; Howlett et al., 1973), and tryphine (pollenkitt), which forms part of the extracellular pollen coat, and mediates the adhesion of the pollen grain to the stigma surface (Dickinson and Lewis, 1973).

The degeneration of the tapetum may also allow the subsequent programmed development of the endothecium, involved in anther dehiscence; as some substances present within the tapetum inhibit the development of endothecium fibrous wall thickenings (De Fossard, 1969), and in male sterile lines examined where the tapetum is persistent, no endothecium thickenings are seen (Chauhan, 1979). Furthermore chemically induced semi male sterile plant lines, generated by spraying with low concentrations of phytagametocidal compounds, showed a delayed degeneration of the tapetal cells, which is accompanied by the delayed formation of endothecium band thickenings (Chauhan, 1979).

Abnormal tapetum development has been identified in some genic male sterile plant lines (1.4) and in some cytoplasmic male sterile lines. Anomalous tapetal behaviour includes premature degeneration of the tapetum, as seen in one cms line of Capsicum annuum (pepper), where the tapetum degenerates during meiosis, becoming highly vacuolate, leading to microspore arrest while the tetrads are still enclosed within the callose coat (Horner and Rodgers, 1974). Delayed tapetal degeneration also occurs, as seen in a line of cms Allium cepa (common onion), where the microspores degenerate after release from the tetrad, whereas the tapetal degeneration doesn't occur until the microspores have shrivelled and died (reviewed in Kaul, 1988).

Abnormal tapetal function has also been visualised in the timing of callose expression. In one mutant line of cms Sorghum, hypertrophy and vacuolation of the tapetal cells is accompanied by premature callose dissolution and male sterility (Overman and Warmke, 1972). Another male sterile cms line of Petunia (Frankel et al., 1969) also shows premature callose expression. Equally, delayed callose
activity, demonstrated by a mutant line of *Petunia* (Izhar and Frankel, 1971) also results in microspore abortion and male sterility. Transgenic tobacco, resulting in the premature dissolution of callose in the anther, have also been generated (Worrall et al., 1992) (1.4.5) and these transgenic lines also demonstrate male sterility as a result.

### 1.3.2 Tapetal-specific gene expression

Anther development involves complex gene expression, required to determine, differentiate and regulate the functions of the specific cell types. Of the 25 000 diverse genes expressed in the anther, 10 000 are estimated to be anther specific (Kamalay and Goldberg, 1980, 1984). The study of anther specific genes, isolated from immature anther specific cDNA libraries, has shown that tapetal messages dominate the population of transcripts within the anther, and their gene expression patterns are both temporally and spatially regulated (Koltunow et al., 1990; Smith et al., 1990; Scott et al., 1991a, 1991b). The high abundance of tapetal specific mRNAs, is explained by the considerable metabolic activity seen in the tapetum, along with a high level of expression of unique set of tapetal-specific genes. In addition to the high tapetal-specific gene expression levels, the sporogenous cells produce only low abundant transcripts, during the initial period of microspore development.

Five clones, *TA26, TA29, TA32, TA13* and *TA36*, isolated from a tobacco immature cDNA library (Koltunow et al., 1990), were shown by in situ hybridisation to be localised to the tapetum. Further in situ hybridisation studies carried out on different anther development stages, along with similarly staged RNA dot blot and RNA gel studies, probed with these clones showed their temporal expression patterns to be consistent with the presence and degeneration of the tapetum. DNA sequencing studies showed *TA32* and *TA36* to share homology with mRNAs encoding lipid transfer proteins. *TA13* and *TA29* showed homology with a group of mRNAs that encoded glycine rich proteins with priorities of cell wall proteins. *TA26* failed to show sequence homology with any known mRNA or protein. Deletion studies carried out on the TA29 5' region, showed the regulation of promoter sequences, to be under transcriptional control between the -279 and -150 region. Further gain of function experiments with a heterologous promoter, identified the -207 to -85 region of the TA29 promoter, necessary and sufficient to drive tapetal-specific gene expression.

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Two cDNA libraries constructed from *B. napus*, were enriched for specific developmental stages, by exploiting the allometric relationship between bud length and developmental stage. The sporogenesis and microspore development libraries was constructed from anthers dissected from 1.2 - 1.8 mm, and 1.8 - 4.0 mm buds respectively (Scott et al., 1991a, 1991b). Anther specific clones isolated by differential screening were selected for further characterisation. The temporal expression patterns were examined by RNA dot blot and RNA gels made from staged *B. napus* buds, and all the clones showed varied but expected patterns of expression depending on the library of origin. Three of the clones isolated from the sporogenesis library A3, A8 and A9, were shown by in situ hybridisation to be tapetal-specific. Later A6, also isolated from the sporogenous library proved to be tapetal-specific as shown by promoter-barnase fusions (Hird et al., 1993). Sequence data from the A8 and A9 clones, implicates these two genes to encode polypeptides that resemble some storage proteins and certain protease inhibitors. Putative signal sequences were also present suggesting the secretion of these polypeptides from the tapetal cells into the locular space.

The A6 and A9 cDNAs from *B. napus* were used to screen an *A. thaliana* genomic library (Roberts et al., 1993). Two *A. thaliana* genomic clones isolated, G61 and G62 (Hird et al., 1993), revealed 96-98 % homology to the *B. napus* A6 cDNA sequence at the nucleotide level, within both coding and upstream regions. A third *A. thaliana* genomic clone isolated, G9.1, showed 76% homology to the *B. napus* A9 cDNA, at the nucleotide level (Paul et al., 1992).

To determine the temporal expression pattern of the A6 gene, a 885 bp promoter fragment from G62, was linked to the *udiA (GUS)* reporter gene (Bevan, 1984) and to the *Bacillus amyloliquefaciens* RNase barnase gene (Paul et al., 1992). Chimaeric promoter-reporter gene constructs were transformed into tobacco and *B. napus*. Histochemical staining of A6-GUS transgenic plants with X-GLUC, showed GUS activity in the tapetum of transformed tobacco anthers and in whole buds of *B. napus* transformants. Additionally, GUS activity was visualised in mature tobacco microspores and pollen, but not in the microspores or pollen of the *B. napus* transformant. Fluorimetric assays demonstrated a sharp peak of GUS activity in tobacco anthers and *B. napus* buds, immediately before microspore release. GUS activity subsequently declined in both plants. Fluorometric data showed that the GUS activity detected in mature microspores and pollen of tobacco is low in comparison with the levels of tapetal GUS activity.
A6-barnase tobacco transformants were completely male sterile but otherwise phenotypically normal. Initially these transformants develop as WT, producing a distinct tapetal layer, synthesis callose to varying degrees and complete meiosis to form tetrads. Following tetrad formation, the tapetal layer stains less densely and degenerates earlier in A6-barnase transgenic plants than WT counterparts, and callose degradation or microspore release fails to occur in transgenic plants. A6-barnase B. napus transformants were completely male sterile, and in addition two out of the four transformants showed floral abnormalities including reduced female fertility. A6-GUS and A6-barnase data demonstrates that the A6 promoter is active in the tapetum after the completion of meiosis, but immediately prior to and declining following microspore release.

The timing of A6 expression, closely matches the expected pattern of callase activity in the anther (Stieglitz, 1977), which is tightly regulated and coordinated with microspore release, shown as aberrant expression leads to male sterility (1.3.1). Therefore temporal and sequence data of the A6 gene, suggests that A6 may encode a component of the callase enzyme complex (Hird et al., 1993).

The spatial and temporal expression patterns of A9, were examined using three different length transcriptional fusions of the A9 promoter, 1437, 934 and 329 bp, to the GUS gene, transformed into N. tabacum. Histochemical staining of transformed anther sections, showed GUS activity to be limited to the tapetal cells. Fluorimetric assays on staged buds from A9-GUS transformants indicated GUS activity first appears in tapetum during early sporogenous cell meiosis, increases dramatically, reaches a plateau and then falls precipitously and ceases in the anthers with pre-mitotic microspores. No differences of GUS activity in the anthers was seen between the three promoter lengths. Additionally, A9-barnase transgenic N. tabacum plants were generated (Paul et al., 1992), which demonstrated that the tapetum had already degraded prior to sporogenous cell meiosis.

Primary sequence of A9, used to search the protein and DNA databases, showed no overall homology, although A9 was found to contain a conserved cysteine motif also present in a diverse group of plant seed proteins (Kreis et al., 1985), possibly involved in the formation of disulphide bridges require for tertiary structure (Maeda et al., 1983).

Comparison of the temporal expression patterns of the A6 and A9 genes using GUS data, would implicate both genes to share the same pattern of
expression, in the tapetum. However comparison of the barnase data indicates that A9 is expressed prior to the A6 gene, because the microsporocytes do not enter meiosis or form callose walls in the A9-barnase tobacco transformants, as they do in A6-barnase. The difference of initial A9 expression shown between the GUS and barnase data, implicates promoter fusions to the RNase barnase gene to be more sensitive indicators of the onset of promoter activity than promoter fusions to GUS.

1.4 Mutants of *A. thaliana* affected in male fertility

To achieve male fertility, stamen differentiation and microsporogenesis are required to follow a genetically coordinated developmental pathway. The complex expression patterns of organ specific and housekeeping genes, along the stamen differentiation pathway results in the formation of the stamens, the production of pollen, containing the male gametes, and the regulation of pollen release and pollen stigma interactions. Mutants of *A. thaliana* which carry lesions in a nuclear gene, resulting in male sterility have been identified at different stages of androgenesis. In structural male sterile mutants, anther or filament morphology is affected, sporogenous male sterile lines carry defects in sporogenesis, microspore development, or microspore maturation and in functional male sterile mutants pollen release or pollen stigma interactions are impaired.

1.4.1 Structural mutants affected in stamen morphology

A number of structural fertility mutants, summarised in the table below, have been identified in *A. thaliana*, where a single genetic lesion disrupts the normal development of the inflorescence meristem and causes male sterility.

<table>
<thead>
<tr>
<th>Gene responsible</th>
<th>Mutant phenotype</th>
<th>Reference</th>
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<tr>
<td>FILAMENTOUS</td>
<td>erratic floral meristem production,</td>
<td>Komaki et al., 1988</td>
</tr>
<tr>
<td>FLOWER (FIL)</td>
<td>few floral organs, lack of anthers</td>
<td></td>
</tr>
<tr>
<td></td>
<td>petaloid sepals/stamenoid petals/</td>
<td></td>
</tr>
<tr>
<td></td>
<td>carpelloid stamens.</td>
<td></td>
</tr>
<tr>
<td>PIN-FORMED (PIN)</td>
<td>forms no floral buds, or occasional defomed flowers.</td>
<td>Goto et al., 1991</td>
</tr>
</tbody>
</table>
Structural mutants affected in stamen morphology (Continued)...

<table>
<thead>
<tr>
<th>Gene responsible</th>
<th>Mutant phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANtherless</td>
<td>normal filaments, anthers absent or show homeotic conversion.</td>
<td>Chaudhury et al., 1992</td>
</tr>
<tr>
<td>UNDEVELOPED</td>
<td>normal filaments, abnormal</td>
<td></td>
</tr>
<tr>
<td>ANther</td>
<td>club shaped anthers which lack pollen - EMS.</td>
<td>Goldberg et al., 1993</td>
</tr>
<tr>
<td>APetala3 (AP3)</td>
<td>homeotic conversion of petals to sepals and stamens to carpels.</td>
<td>Bowman et al., 1989</td>
</tr>
<tr>
<td>PISTILLATA (PI)</td>
<td>homeotic conversion of petals to sepals and stamens absent or converted to carpels, abnormal filamentous gynoecium.</td>
<td>Bowman et al., 1989</td>
</tr>
<tr>
<td>AGamous (AG)</td>
<td>homeotic conversion of stamens to petals, no gynoecium, indeterminate floral meristem.</td>
<td>Hill and Lord, 1989</td>
</tr>
</tbody>
</table>

1.4.2 Sporogenous mutants affected in microgametogenesis

1.4.2.1 Pre-meiotic and meiotic mutants

Male sterile mutants of *A. thaliana*, listed below, have been isolated which show pre-meiotic and meiotic defects in sporogenesis which lead to pollen abortion and male sterility.

<table>
<thead>
<tr>
<th>Gene responsible</th>
<th>Mutant phenotype</th>
<th>mutagen</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS3</td>
<td>pre-meiotic arrest of sporogenous cell development.</td>
<td>EMS</td>
<td>Chaudhury et al., 1994</td>
</tr>
<tr>
<td>MS4</td>
<td>meiocytes arrest at dyad stage, by end of meiosis I.</td>
<td>EMS</td>
<td>Chaudhury et al., 1994</td>
</tr>
</tbody>
</table>
Pre-meiotic and meiotic mutants (Continued)...

<table>
<thead>
<tr>
<th>Gene responsible</th>
<th>Mutant phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MS5</strong></td>
<td>pre-meiotic or meiotic arrest of meiocytes.</td>
<td>EMS</td>
</tr>
<tr>
<td><strong>MS15</strong></td>
<td>pre-meiotic arrest of meiocyte development.</td>
<td>EMS</td>
</tr>
<tr>
<td><strong>7219</strong></td>
<td>asynchronous and varied differentiation pathways of microsporogenesis, no callose production, female sterile.</td>
<td>T-DNA</td>
</tr>
<tr>
<td><strong>7593</strong></td>
<td>asynchronous and varied differentiation pathways of microsporogenesis, no callose production</td>
<td>T-DNA</td>
</tr>
<tr>
<td><strong>6492</strong></td>
<td>meiosis extended, arrest at tetrad stage each tetrad containing up to 8 microspores, female sterile.</td>
<td>T-DNA</td>
</tr>
<tr>
<td><strong>MEI-1</strong></td>
<td>arrest at tetrad stage each tetrad containing 5 to 8 microspores.</td>
<td>T-DNA</td>
</tr>
<tr>
<td><strong>TETRASPORE</strong></td>
<td>failure of cytokinesis at the end of meiosis.</td>
<td>EMS/T-DNA</td>
</tr>
<tr>
<td><strong>MS37 (MSY)</strong></td>
<td>abnormal meiosis, reduced callose.</td>
<td>EMS</td>
</tr>
<tr>
<td><strong>MS32 (MSW)</strong></td>
<td>abnormal meiosis, reduced callose.</td>
<td>EMS</td>
</tr>
<tr>
<td><strong>MS31 (MSK)</strong></td>
<td>normal meiosis, reduced callose.</td>
<td>EMS</td>
</tr>
</tbody>
</table>
1.4.2.2 Post-meiotic mutants

A number of male sterile mutants have been isolated in *A. thaliana*, listed below, were meiosis is normal and pollen abortion occurs at a later stage in development.

<table>
<thead>
<tr>
<th>Gene responsible</th>
<th>Mutant phenotype</th>
<th>mutagen</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MS1</em></td>
<td>microspore arrest following microspore release, failure in pollen wall development.</td>
<td>EMS</td>
<td>Veen and Wirtz, 1968</td>
</tr>
<tr>
<td><em>MS7</em></td>
<td>microspore arrest following microspore release.</td>
<td>-</td>
<td>Chaudhury, unpublished results</td>
</tr>
<tr>
<td><em>MS8</em></td>
<td>microspore arrest following microspore release.</td>
<td>-</td>
<td>Chaudhury, unpublished results</td>
</tr>
<tr>
<td><em>MS2</em></td>
<td>microspore arrest following microspore release.</td>
<td><em>I</em>- element</td>
<td>Aarts et al., 1993</td>
</tr>
<tr>
<td><em>APT</em></td>
<td>few abnormal tetrads, microspore arrest following microspore release.</td>
<td>EMS</td>
<td>Regan and Moffatt, 1990</td>
</tr>
<tr>
<td><em>MS33 (MSZ)</em></td>
<td>no filament elongation, abnormal globular tapetum showing late degeneration, pollen decays at late stage.</td>
<td>EMS</td>
<td>Dawson et al., 1993</td>
</tr>
<tr>
<td><em>MS38</em></td>
<td>filament elongation delayed, abnormal tapetum, pollen decays at late stage.</td>
<td>gamma</td>
<td>Mulligan et al., 1994</td>
</tr>
<tr>
<td><em>MS36</em></td>
<td>pollen wall development fails, pollen decays at late stage.</td>
<td>gamma</td>
<td>Mulligan et al., 1994</td>
</tr>
<tr>
<td><em>MS34</em></td>
<td>pollen grains of various sizes decay at late stage.</td>
<td>gamma</td>
<td>Mulligan et al., 1994</td>
</tr>
<tr>
<td><em>COI1</em></td>
<td>shorter stamens than WT, pollen decays at late stage.</td>
<td>EMS</td>
<td>Feys et al., 1994</td>
</tr>
</tbody>
</table>
1.4.3 Functional mutants affected in dehiscence

Two mutant lines of *A. thaliana*, indicated below, produce viable pollen but remain male sterile due to their inability of incorrect timing of pollen release.

<table>
<thead>
<tr>
<th>Gene responsible</th>
<th>Mutant phenotype</th>
<th>mutagen</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MS35 (MSH)</em></td>
<td>incomplete dehiscence no pollen released.</td>
<td>X-ray</td>
<td>Dawson et al., 1993</td>
</tr>
<tr>
<td><em>LATE</em></td>
<td>dehiscence occurs after the DEHISCENCE stigma is no longer receptive to pollination.</td>
<td>EMS</td>
<td>Goldberg et al., 1993</td>
</tr>
</tbody>
</table>

1.4.4 Functional mutants affected in pollen stigma interactions

Mutations in the *ECERIFERUM (CER)* genes disrupts different steps in the wax biosynthesis pathway, and generates mutants with a bright green appearance. Some of these mutants, listed in the table below, also demonstrate male sterility due to the inability of the pollen they produce to germinate on receptive stigmas.

<table>
<thead>
<tr>
<th>Gene responsible</th>
<th>Mutant phenotype</th>
<th>mutagen</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>CER1</em></td>
<td>abnormal lipid dispersion in tryphine layer surrounding pollen grains.</td>
<td>/- element</td>
<td>Aarts et al., 1995</td>
</tr>
<tr>
<td><em>CER6 (POP1)</em></td>
<td>lack of tryphine surrounding pollen grains.</td>
<td>ethyl-nitrosourea</td>
<td>Preuss et al., 1993</td>
</tr>
</tbody>
</table>
1.4.5 Genetically engineered male sterility and its applications

Many flowering plants are hermaphrodites, possessing both the male and female reproductive organs on the same plant, thus the majority of seed produced arises via self-pollination. Genetic engineering has been utilised to induce male sterility, in hermaphrodite plants, to enable directed crosses between inbred lines for the production of hybrid seed stocks. Hybrid varieties are desirable because some show increased vigor, yield, uniformity and also increased resistance to disease and environmental stress. A genetic system of induced male sterility and restoration would eliminate the requirement for manual emasculation, or the use of self-incompatibility. Since the majority of male sterile plant lines show tapetal abnormalities (Kaul, 1988), and a number of tapetal specific genes had been isolated (Koltunow et al., 1990; Smith et al., 1990; Scott et al., 1991a, 1991b) due to their high abundance, it was realised that the genetically engineered destruction of this specialised cell type, would provide a direct route to male sterility.

A system for male fertility control, developed by Plant Genetic Systems in Gent, Belgium in conjunction with R.B. Goldberg's laboratory in California, utilises the tapetal-specific TA29 tobacco gene promoter (Koltunow et al., 1990; Mariani, 1990, 1992), and the barnase/barstar defence system of the bacterium Bacillus amyloliquefaciens (Hartley, 1989). Barnase is an extracellular RNase inhibitor produced by the bacteria to defend itself against microbial attack, and barstar is the RNase-inhibitor that the bacteria uses intracellularly to protect itself from the cytotoxic effects of barnase (Hartley, 1989). Genetic engineered male sterility has been induced by the transgenic expression of the TA29 promoter fused to the barnase gene, which causes the selective destruction of the tapetum and leads to the production of male sterile plants (Mariani et al., 1990). Male fertility is restored by the introduction of the TA29 promoter driving the barstar gene coding region. Thus when a male sterile TA29 barnase plant is crossed with a male fertile TA29 barstar, the progeny inherit both transgenes. Such plants develop an intact tapetum resulting in fertile pollen production (Mariani et al., 1992). This system presents a solution that enables the large scale production of fertile hybrid seeds that can be utilised in a wide variety of monocot and dicot plant species, due to the correct regulation of the TA29 promoter in many plant species (Mariani et al., 1992).

The TA29 gene promoter has similarly been used to induce male sterility in tobacco, by destruction of the tapetum and sporogenous cells, when fused to the diphtheria toxin A chain gene (Koltunow et al., 1990). Another tapetal-specific gene
promoter, A9, has been fused to the temperature sensitive diphtheria toxin A chain, A9-DTAtsm, (Guerineau et al., in press). The Arabidopsis lines carrying the A9-DTAtsm transgene were male sterile at 18 °C and male fertile at 25 °C. Examination of male sterile anthers, carrying the transgene, indicated that the tapetum had not been completely destroyed but had undergone discrete alterations, indicated by reduced staining with toluidine blue, attributed to a lower ribosome density visualised through electron microscopy (Guerineau et al., in press).

Other strategies for the induction of male sterility include the tapetum-specific A9 and A3 gene promoters fused to a pathogenesis-related β-1,3-glucanase gene, causing the premature dissolution of callose from around the meiocytes prior to meiosis. Although these male sterile transgenic tobaccos continue development through meiosis, to produce tetrads of microspores, they are not held together by callose, and the tetrads adhere to each other and form clumps. Microspore release does take place in these transgenic lines, however the free microspores eventually burst (Worrall et al., 1992). The A6 and the A9 tapetal-specific promoters have also been shown to cause the selective destruction of the tapetum, when fused to the barnase gene (Paul et al., 1992; Hird et al., 1993).

Male sterility can also be induced by the tapetal-specific expression of a Chimaeric Agrobacterium rhizogenes rolB gene, that increases auxin activity in the anther and causes developmental abnormalities (Spena et al., 1992). These additional tapetum specific promoter fusions to disrupter genes, causing the dysfunction or ablation of the tapetum are adequate to induce male sterility, but they lack the restorer element required for seed production in the hybrid progeny, especially important if the seed is the harvested crop. The restoration of male fertility, for such future systems, may be achieved by anti-sense RNA, or ribosomes directed to the disrupter gene, or where available a protein inhibitor, or protease (Scott et al., 1991b). Even more desirable, than a two element system, would be the induction of male sterility through a single transgene, only after the application of an external stimulus, although problems of “leaky” genes, resulting in the production of some pollen, must be overcome first (Scott et al., 1991b).
1.5 The scope of this thesis

The molecular genetic characterisation of floral mutants has revealed some of the regulatory processes involved in the establishment of the floral meristem and the specification of floral organ position and identity. In addition to the other floral organs studied, a number of genes expressed in the anther have been characterised, and these fall into two main groups, pollen-specific genes and tapetum-specific genes.

However, little is known about how stamen tissue differentiation is controlled at the genetic level. The role of AP3 and AG has been proposed in the initiation or control of stamen identity in A. thaliana (1.1), and these genes act either directly or indirectly through a cascade of gene expression that leads to the eventual expression of tapetal-specific genes, such as A6 and A9 (1.3). The main focus of the work described in this thesis is to bridge this gap in development, and identify and characterise genes involved in early stamen histodifferentiation. The strategy used to achieve this goal, was to split the stamen differentiation pathway into defined temporal windows using the tapetum-specific promoters, A6 and A9 cloned from A. thaliana, and then to screen for plants within a mutated population, that carry lesions in specific windows by assaying for the activity/inactivity of reporter genes fused to the promoters.

Therefore the main aim of this work is to generate and screen a suitable mutagenised population of A. thaliana and characterise any novel mutants isolated. Initial studies were therefore required to further evaluate the temporal expression patterns of A6 and A9, by chimaeric fusions to suitable reporter genes, GUS and LUC. Also investigated, is the proposal that mutants arising from this screen could be identified visually by fusing one of the tapetal-specific promoters to the TMS2 gene, avoiding laborious and time consuming repetitions of reporter gene assays. Additional work in this thesis is the 5' deletion analysis of the A9 promoter investigated in tobacco, and the effects of expressing the AG gene in the tapetum of A. thaliana transformants.
Fig. 1.1 The histology of the microsporangium in *A. thaliana*.

Abbreviations: **E**, epidermis; **En**, endothecium; **Mi**, middle layer; **Ta**, tapetum; **S**, sporogenous cells; **C**, connective tissue.
Fig. 1.2 Microgametogenesis in *B. napus*, showing the cytological changes that occur within the sporogenous cells and tapetum during pollen formation.

Abbreviations: **A**, archesporial cell; **PP**, primary parietal cell; **Ta**, tapetal cell; **PS**, primary sporogenous cell; **S**, sporocyte; **M**, meiocyte; **Me**, meiosis; **T**, tetrad; **MR**, microspore release; **M1**, microspore interphase; **DM**, dinucleate microspore; **TP**, trinucleate pollen.

Red shading indicates callose; yellow shading indicates sporopollenin.
Sporogogenesis

Microspore development

Pollen maturation
Chapter 2
Materials and Methods
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abs</td>
<td>absorbance</td>
</tr>
<tr>
<td>AG/ag</td>
<td>agamous gene/mutagenised agamous gene</td>
</tr>
<tr>
<td>AG</td>
<td>agamous protein/enzyme</td>
</tr>
<tr>
<td>AMPS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>A. thaliana</td>
<td><em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>A. tumefaciens</td>
<td><em>Agrobacterium tumefaciens</em></td>
</tr>
<tr>
<td>BDMA</td>
<td>benzyldimethylamine</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>β-Me</td>
<td>beta-mercaptoethanol</td>
</tr>
<tr>
<td>B. napus</td>
<td><em>Brassica napus</em></td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CIP</td>
<td>calf intestinal phosphatase</td>
</tr>
<tr>
<td>2,4-D</td>
<td>2,4-dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytosine triphosphate</td>
</tr>
<tr>
<td>DDSA</td>
<td>dodecenyl succinic anhydride</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanidine triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxynucleotides</td>
</tr>
<tr>
<td>dH_2O</td>
<td>sterilised double distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase activity/protein/enzyme</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase gene</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>IAA</td>
<td>indole-3-acetic acid</td>
</tr>
<tr>
<td>IAM</td>
<td>indole-3-acetamide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>2-ip</td>
<td>2-isopentenyladenine</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalatosidase</td>
</tr>
<tr>
<td>KAC</td>
<td>potassium acetate</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pair</td>
</tr>
<tr>
<td>KoAC</td>
<td>potassium acetate</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>LUC</td>
<td>luciferase activity/protein/enzyme</td>
</tr>
<tr>
<td>molar</td>
<td></td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μl</td>
<td>microlitre</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
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<tr>
<td>min</td>
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<tr>
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<td>millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>ms</td>
<td>male sterile</td>
</tr>
<tr>
<td>4-MU</td>
<td>4-methyl umbelliferone</td>
</tr>
<tr>
<td>MUG</td>
<td>4-methyl umbelliferyl-β-D-glucuronide</td>
</tr>
<tr>
<td>NAM</td>
<td>napthalene acetamide</td>
</tr>
<tr>
<td>NAA</td>
<td>napthalene acetic acid</td>
</tr>
<tr>
<td>N₂</td>
<td>nitrogen</td>
</tr>
<tr>
<td>N. tabacum</td>
<td><em>Nicotiana tabacum</em></td>
</tr>
<tr>
<td>OD</td>
<td>optical density (measured in nm)</td>
</tr>
<tr>
<td>o/n</td>
<td>overnight</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>S/N</td>
<td>supernatant</td>
</tr>
</tbody>
</table>
TEMED  N,N,N',N'-tetramethyl-ethylenediamine
U      enzyme units
UV     ultraviolet
v/v    volume to volume ratio
V      volts
W      watts
WT     wild type
w/v    weight to volume ratio
X-GAL  5-bromo-4-chloro-3-indolyl-β-D-galactoside
X-GLUC 5-bromo-4-chloro-3-indolyl-β-D-glucuronide
2.1 Chemicals and reagents

Chemicals and reagents were obtained from Agar Scientific, BDH, Boehringer Mannheim, Difco Laboratories, Fisons, Kodak, New England Biolabs, Pharmacia, Promega, Sigma and Stratagene. Deionised water was obtained from an Elga-stat Option 2 water purifier (ELGA). Oligonucleotide primers were synthesised at Leicester University in the Biochemistry Department.

2.2 Bacterial strains - Growth and storage conditions

2.2.1 Bacterial strains

*E. coli* XL1-blue: recA1, gyr A96, thi, hsd R17, sup E44, re1A1, lac [F', pro AB, laq 1q, ZΔM15], Tn10 (tetR) (Bullock et al., 1987).

*E. coli* JM83: F-, ara, A (lac-proAB), rpsL, ø80dlaq ZΔM15 (Yanish-Perron et al. 1985)

*E. coli* JM101: sup E, thi, A (lac-proAB), [F', tra D36, pro AB, lac ZΔM15] (Yanish-Perron et al. 1985)

*A. tumefaciens* host strain (LBA4404): rifR, strR (Hoekema et al., 1983).

2.2.2 Bacterial media

<table>
<thead>
<tr>
<th>Luria-Bertani (LB)</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>bactotryptone</td>
<td>10</td>
</tr>
<tr>
<td>NaCl</td>
<td>10</td>
</tr>
<tr>
<td>yeast extract</td>
<td>5</td>
</tr>
</tbody>
</table>

LB solid media made as above, with 1.5% technical agar no.3

<table>
<thead>
<tr>
<th>NZY</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>caesine hydrolysate</td>
<td>10</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
</tr>
<tr>
<td>yeast extract</td>
<td>5</td>
</tr>
<tr>
<td>MgSO4.H2O</td>
<td>2</td>
</tr>
</tbody>
</table>

NZY solid media made as above, with 1.5% technical agar no. 3

<table>
<thead>
<tr>
<th>2XYT</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>bactotryptone</td>
<td>16</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
</tr>
<tr>
<td>yeast extract</td>
<td>10</td>
</tr>
</tbody>
</table>

30
media adjusted to pH7 using NaOH
2XYT solid media made as above, with 0.8% Difco Bacto Agar
After preparation all media were sterilised by autoclaving.

2.2.3 Culture conditions

Bacterial cultures were grown from glycerol stocks or from individual colonies. Bacteria were grown in 5 ml of liquid broth in sterile universals, or 50 ml of liquid both in 250 ml flasks, containing appropriate antibiotic selection, indicated below in μg/ml:-

<table>
<thead>
<tr>
<th></th>
<th>E. coli</th>
<th>A. tumefaciens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin (kan)</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Ampicillin (amp)</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>streptomycin (strep)</td>
<td>-</td>
<td>200</td>
</tr>
<tr>
<td>augmentin (aug)</td>
<td>-</td>
<td>400</td>
</tr>
<tr>
<td>cefotaxime (cefo)</td>
<td>-</td>
<td>200</td>
</tr>
<tr>
<td>Rifamphicin (rif)</td>
<td>-</td>
<td>50</td>
</tr>
</tbody>
</table>

All liquid cultures were grown in a shaking incubator, E.coli cultures were incubated at 37 °C, for 4 - 6 hr or o/n, and A. tumefaciens cultures were incubated at 28 °C for 24 hr. Bacteria were also grown on solid agar plates, containing selection, by streaking with a sterile loop or by spreading with a sterile glass rod. Inverted plates were then incubated o/n (48 hr for A. tumefaciens), at the appropriate temperature.

2.2.4 Preparation of glycerol stocks

Bacterial strains and clones were kept at -80 °C, in glycerol for long term storage. Bacterial clones were streaked on to solid media plates and a single colony was grown in 5 ml of liquid media. Plasmids were checked by small scale DNA preparation and restriction enzyme digestion. Once authenticated, 1 ml of culture was added to a 1.5 ml, sterile screwcap tube which contained 0.5 ml of sterile glycerol. The tube was inverted to form a homogeneous mixture and then flash frozen in liquid nitrogen, before being transferred for long term storage at -80 °C. Bacterial liquid cultures and solid plates were stored in the short term at 4 °C.
2.3 Preparation of bacterial DNA

2.3.1 Small scale preparation of plasmid DNA from *E. coli*

Method described by Bimboim and Doly (1979). 1.5 ml taken from a 5 ml o/n culture was spun down in a microcentrifuge tube at 14 000 g, for 5 min. Bacterial cells were resuspended, by vortexing, in 100 µl of lysis buffer (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA, freshly prepared lysozyme was added to a final concentration of 1 mg/ml). 200 µl of alkiline-SDS (0.2 M NaOH, 1% SDS) was added, and the tube was inverted until the solution cleared. 150 µl of 3 M KAc was added, and the tube was mixed gently. Insoluble cell debris was precipitated by centrifugation at 14 000 g for 10 min, and the clear S/N of cell extract was transferred to a fresh tube. An equal volume of 25:24:1 v/v phenol:chloroform:isoamyl-alcohol was added to the S/N and vortexed to remove proteins. The resulting emulsion was centrifuged at 14 000 g for 2 min and the upper aqueous phase was transferred to a fresh tube. Nucleic acids were precipitated by the addition of 2 volumes of ethanol. Pellets were washed with 100 µl of 70% ethanol, vacuum dried and resuspended in 50 - 100 µl of dH2O. Plasmid preparations were stored at -20 °C. Alternatively Wizard™ preparations of nucleic acids were carried out using resin and columns supplied by Promega. 1.5 ml of culture was spun down at 14 000 g, for 5 min, resuspended in 200 µl of cell resuspension solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 µg/ml RNase A), 200 µl of cell lysis solution (0.2 M NaOH, 1% SDS) was added, mixed by inversion and 200 µl of neutralisation solution (1.32 M KAc) was added. After centrifugation at 14 000 g for 10 min the S/N was removed to a fresh microcentrifuge tube and 1 ml of Wizard™ DNA purification resin was added and mixed well. Using a 2 ml syringe attached to a Wizard™ minicolumn, the DNA/resin mix was transferred to the minicolumn, 2 ml of column wash (100 mM NaCl,10 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 50 % ethanol) were passed through the column to wash the DNA. The column was inserted into the top of a 1.5 ml microcentrifuge tube and spun in a centrifuge for 20 secs, at 14 000 g, to remove any residual column wash solution. The column was inserted into a fresh microcentrifuge tube and 50 µl of dH2O, preheated to 65 °C was added to dissolve the nucleic acids. Nucleic acids were eluted by centrifugation for 20 secs at 14 000 g.
2.3.2 Large scale preparation of plasmid DNA from E. coli

Large scale plasmid DNA preparations were carried out on 50 - 500 ml o/n cultures. The method used is an unpublished protocol of R. Treisman, and is based on the methods described by Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). 500 ml of an o/n bacterial culture was spun down in two 250 ml centrifuge bottles at 4 000 rpm for 5 min, in a Sorvall rotor. The S/N was discarded and the pellet was resuspended, by vortexing, in 10 ml of lysis buffer. 20 ml of alkaline-SDS was added, and the solution was inverted until the solution cleared. 15 ml of 3M K0AC was added, to precipitate chromosomal DNA, high molecular weight RNA and protein. The bacterial lysate was spun for 15 min at 4 000 rpm at 4 °C. The S/N was filtered through 4 layers of cheesecloth, into 250 ml bottles. 0.6 volume of isopropanol was added, mixed well and stored at RT for 10 min. Nucleic acids were pelleted by centrifugation at 5 000 rpm for 15 min at RT. The pellets were washed with 70 % ethanol, allowed to dry and resuspended in 3 ml of TE (pH 8). To precipitate remaining high molecular weight RNA, an equal amount of 5 M LiCl was added, mixed and spun at 10 000 rpm for 10 min at 4 °C. The S/N was transferred to a fresh corex tube and an equal volume of isopropanol was added and mixed. Nucleic acids were precipitated by centrifugation at 10 000 rpm for 10 min at RT. The pellet was washed with 70 % ethanol and allowed to dry. The pellet was resuspended in 500 µl of TE (pH 8), containing DNAase-free pancreatic RNAase (20 µg/ml). The solution was transferred to a microcentrifuge tube and stored at RT for 30 min. 500 µl of 1.6 M NaCl, containing 13 % PEG 8000 was added and mixed. Plasmid DNA was pelleted by centrifugation at 14 000 g for 10 min in a microcentrifuge. The pellet was dissolved in 400 µl of TE (pH 8), extracted once with phenol, once with phenol:chloroform and once with chloroform. The aqueous phase was transferred to a fresh microcentrifuge tube and 100 µl of 10 M ammonium acetate and 800 µl of ethanol was added, mixed well and stored at RT for 10 min. Plasmid DNA was pelleted by centrifugation at 14 000 g for 10 min. The pellet was washed with 70 % ethanol, allowed to dry and resuspended in 500 µl of TE (pH 8). The OD260 of a 1:100 dilution of the plasmid in TE (pH 8) was measured, and the concentration of the plasmid DNA was calculated. 1 OD260 = 50 µg of plasmid DNA/ml (Sambrook et al., 1989). Plasmid DNA samples were stored at -20 °C.
2.3.3 Preparation of plasmid DNA from *A. tumefaciens*

Based on the method described by Birnboim and Doly (1979) for small scale DNA preparations (as described above), this method was used to check *A. tumefaciens* transformants. Modified as follows: bacterial cells were incubated in lysis solution for 30 min at 37 °C; 2 phenol-chloroform extractions were performed on the S/N, the DNA pellet was resuspended in 10 µl and diagnostic restriction digests were carried out on 10 µl samples.

2.4 Manipulation of bacterial DNA

2.4.1 Agarose gel electrophoresis

DNA fragments were separated, visualised and sized by agarose gel electrophoresis. 0.8 - 2 % agarose (Sigma or Kodak) gels, made with 1 x TAE (containing 1 µl of 10 mg/ml ethidium bromide/50 ml) were run in tanks (Bio-Rad), containing 1 x TAE buffer. 10 % loading buffer was added to DNA samples prior to pipetting into wells. 1 kb DNA ladder was run adjacent to samples, to allow fragments to be sized.

| 10 x TAE g/L | loading buffer % |
| tris base 48.44 | xylene cyanol 0.25 |
| EDTA 3.72 | bromophenol blue 0.25 |
| adjust to pH 8 with | glycerol 30 |
| glacial acetic acid | |

2.4.2 Visualisation of nucleic acids

DNA bands were visualised in agarose gels, using ultraviolet-light which causes the ethidium bromide which has intercalated into the double stranded DNA to fluoresce. DNA fragments for cloning, making probes etc. were cut from gels using a scalpel blade and purified as described in 2.4.6.

2.4.3 Restriction enzyme digestion

Restriction enzymes were obtained from Boehringer Mannheim, Gibco-BRL, New England Biolabs and Promega. Enzymes were supplied with their own 10 x incubation buffer. Samples of plasmid DNA were incubated in 1 x enzyme reaction buffer - usually at 37 °C, with appropriate enzyme(s). Volumes of enzyme digestion reactions were typically between 10 - 50 µl. Samples were incubated for 1 - 3 hr or
o/n. 1 μl of 50 μg/ml RNase was incubated with each sample for the last 10 min to degrade any RNA. Digested DNA samples were either visualised on agarose gels or precipitated prior to further manipulation. BclI restriction enzyme digests, for CAPS analysis, were incubated at 50 °C.

2.4.4 Generation of blunt ends using T4 DNA polymerase

In some cases, to facilitate cloning experiments, vectors and DNA fragments, cut with restriction enzymes, which left 3' and 5' overhangs were blunted. After enzymatic digestion to cut the DNA, sample volumes were made up to 100 μl with dH2O. An equal volume of phenol-chloroform was added, vortexed for 30 secs and spun at 14 000 g for 2 min. The S/N was transferred to a fresh microcentrifuge tube and 1/20th of 5 M NaCl and 2.5 volumes of absolute ethanol was added. Samples were incubated at -20 °C for 10 min and spun for 10 min at 14 000 g to precipitate the DNA. Pellets were washed with 100 μl of 70 % ethanol, vacuum dried and resuspended in 13 μl dH2O. 0.5 μl of T4 polymerase, 1.5 μl of 10 x T4 polymerase buffer (both from Sigma) and 1 μl of dNTPs were added. Samples were incubated at 37 °C for 30 min, after which time DNA samples were run on a gel or precipitated once again prior to further analysis.

2.4.5 Dephosphorylation of 5'-terminus

Occasionally cloning experiments required the insertion of a fragment into a plasmid vector restricted at a single site. In order to prevent the vector religating back to itself, calf intestinal, alkaline phosphatase was incubated with the vector catalysing the removal of 5' phosphate groups thereby preventing self-ligation (Sambrook et al. 1989). Digested DNA samples were extracted with phenol-chloroform and ethanol precipitated as described above (2.4.4). DNA pellets were resuspended in 12.5 μl dH2O and 1.5 μl of 10 x CIP buffer and 1 μl of CIP was added. Samples were incubated at 37 °C for 1 hr. After incubation vectors were again phenol-chloroform extracted, ethanol precipitated and resuspended in a small volume, appropriate for use in a ligation reaction, usually 5 - 10 μl of dH2O.

2.4.6 Purification of DNA fragments from agarose gels

DNA fragments were purified from gel slices either by electroelution (2.4.6.1) or use of a GeneClean kit (2.4.6.2).
2.4.6.1 Electroelution

Gel slices were placed in dialysis membrane (Scientific Industries International inc.), in 250 μl of 1 x TE. Dialysis was carried out, for 30 min at 100 V, in an electrophoresis tank containing 0.5 x TBE (10 x TBE = 0.9 M Tris-borate, 0.01 M EDTA). The positive and negative terminals on the powerpack were then reversed for 0.5 min, to allow the DNA adhered to the membrane to be released. The DNA in TE was pipetted up and down the tubing to ensure none had stuck to the sides, and then extracted once with phenol-chloroform and ethanol precipitated as described above (2.4.4). DNA was resuspended in 10 μl of dH₂O.

2.4.6.2 GeneClean

Gel slices were weighed and 300 % (weight/volume) of 3 M NaI was added to the gel slice. Samples were incubated in microcentrifuge tubes, at 60 °C for 5 min, or until the gel slice was dissolved. 10 μl of Glassmilk was added, and the solution was vortexed and incubated on ice for 5 - 15 min. The glassmilk was then pelleted in a centrifuge for 20 secs, and washed with 0.5 ml of New Wash (50 % ethanol; 0.1 M NaCl; 10 mM Tris-HCl, pH 7.5; 1 mM EDTA). The pellet was washed twice more before being resuspended in 10 μl of dH₂O. Samples were incubated for 5 min between 45 - 55 °C and then spun for 1 min at 14 000 g. The S/N containing the DNA was removed to a fresh microcentrifuge tube, and was then ready for ligation reactions, used as probe stocks, or for other manipulations.

2.4.7 Ligation of DNA fragments

DNA fragments with compatible or blunt ends, were ligated together in small volumes containing 1 x ligation buffer (0.5 M Tris-HCl, 10 mM ATP, 100 mM MgCl₂ and 100 mM DTT), 25% PEG and 1U of T4 DNA ligase. The molar ratio of vector to insert used, was 1:1 or 1:3. Incubation temperature and times are indicated below:

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>48</td>
</tr>
<tr>
<td>15</td>
<td>12 (or o/n)</td>
</tr>
<tr>
<td>25</td>
<td>1</td>
</tr>
</tbody>
</table>

Ligated plasmid DNA was ethanol precipitated and resuspended in 1 - 2 μl of
dH₂O. DNA samples were then transformed, using electroporation, into competent cells of an appropriate bacterial host strain.

2.5 Bacterial transformation

2.5.1 Preparation of *E. coli* competent cells

A 250 ml flask containing 100 ml of 2 x YT, was inoculated with 1 ml from a 5 ml culture of JM83, grown in 2 x YT o/n (initially inoculated from a single colony or taken from a glycerol stock). The 100 ml culture was grown to an OD of 0.5 - 1 (Abs₆₀₀). The flask was chilled on ice for 15 min and then spun at 3 000 g for 15 min. Bacterial cells were resuspended in 100 ml of ice cold dH₂O. Cells were spun down and resuspended, as before, first into 50 ml of ice cold dH₂O, then into 2 ml of cold 10 % glycerol and finally into 300 µl of cold 10 % glycerol. 40 µl aliquots were flash frozen in liquid nitrogen and stored at -80 °C, until use.

2.5.2 Preparation of *A. tumefaciens* competent cells

A single colony or glycerol stock of LBA4404 was used to inoculate 5 ml of 2 x YT containing streptomycin at a concentration of 500 µg/ml. The culture was grown for 48 hr at 28 °C. Bacterial cells were spun down for 2 min at 14 000 g, and washed 4 times with 5 ml of cold dH₂O. Cells were then spun again as before resuspending in 0.5 ml of cold 10 % glycerol, before a final spin, to resuspend in 40 µl of cold 10 % glycerol. If not used immediately cells were flash frozen and stored at -80 °C.

2.5.3 Bacterial transformation using electroporation

An aliquot of electroporation competent bacterial cells was placed on ice to thaw. 1 - 2 µl of salt-free ligation reaction, or plasmid was incubated with the cells for 1 or 2 min. Cells were then pipetted into a cold electroporation cuvette (Bio-Rad), across which 2.5 KVolt was discharged using a Bio-Rad electroporator unit, (capacitance 25 µFD, resistance 200 Ω). Cells were incubated, shaking at 37 °C with 1 ml of prewarmed 2 x YT broth, before being plated out on to agar plates, containing appropriate selection.
2.6 Identification of recombinant colonies

2.6.1 a-Complementation colour selection

Cloning vectors capable of α-complementation contain regulatory sequences and coding sequence the first 146 amino acids of the β-galactosidase gene (lacZ). Embedded in frame, within this coding region, is the multiple cloning site into which foreign DNA can be inserted. Non-recombinant vectors of this kind, when used with host cells, which are deletion mutants for the operator-proximal segment of the lacZ gene, can form enzymatically active β-galactosidase protein. Non-recombinant clones were visualised as blue colonies in the presence of the chromogenic substrate X-gal. If however, a fragment of DNA was inserted into the multiple cloning site, the resulting plasmid was not capable of α-complementation, and the resulting recombinant colony was white. Bacteria to be tested for α-complementation were spread with a sterile glass rod on to solid agar plates, containing IPTG and X-gal:- 100 ml of solid bacterial media was melted and allowed to cool. 100 μl of both 25 % IPTG and 50 mg/ml X-gal were added, 25 ml of media was poured per 9 cm diameter petri dish and allowed to set. Serial dilutions of the bacteria were then spread on to the plates and incubated o/n at 37 °C. White colonies were then analysed by colony screening and/or small scale DNA minipreparations and restriction enzyme digestion.

2.6.2.1 Colony screening

Numerous colonies can be screened for recombinant clones using radioactive probes. Individual colonies, picked off agar plates using sterile tips, were dotted on to a master agar plate, and correspondingly on to a hybond membrane (Amersham). Plates were incubated at 37 °C o/n. Hybond membranes were placed bacteria side up, on 3MM filter paper soaked in 2 x SSC and 0.5 % SDS, for 2.5 min and then placed in the microwave on full power for 2.5 min to cross-link bacterial DNA to the membrane.

2.6.2.2 Preparation and radioactive labelling of probe stock

DNA for radioactive probe stocks, was appropriately digested with restriction enzymes, run on an agarose electrophoresis gel and appropriate fragments were cut and eluted from the gel slice (2.4.6). The DNA sample was boiled for 5 min to generate single stranded template, which was used in the oligolabelling reaction:-
OLB (buffer - Draper et al., 1988;  
plus dNTPs)  3 µl  
BSA (10mg/ml)  1 µl  
Klenow enzyme  0.6 U  
DNA template  9.4 µl  
\(^{32}\)P dCTP (370MBq/ml)  1 µl

Samples were incubated at 37 °C, for 1 hr. Radiolabelled probe stocks were purified, by spinning for 2 min at 1000 rpm, through a packed Sephadex G50 (Sigma) column. Unincorporated \(^{32}\)P dCTP was retained in the column and larger radiolabelled cDNA fragments were eluted. % incorporation of \(^{32}\)P dCTP was measured using a scintillation counter.

2.6.2.3 Prehybridisation and hybridisation of probe

Hybond filters were incubated at 65 °C, for 1 hr, in 25 ml of prehybridisation solution (0.5 % SDS, 5 X SSPE, 5 X Denharts, 20 µg/ml sheared herring sperm DNA).

\[
\begin{align*}
20 \times \text{SSPE} & \quad 50 \times \text{Denharts (stored at -20 °C)} \\
3.6 \text{ M NaCl} & \quad 1 \% \text{ BSA} \\
0.2 \text{ M NaH}_2\text{PO}_4 & \quad 1 \% \text{ P.V.P.} \\
0.02 \text{ M EDTA} & \quad 1 \% \text{ Ficoll 400}
\end{align*}
\]

The radiolabelled probe was heated at 100 °C, for 5 min before being added to the prehybridisation solution and incubated for 3 - 4 hr or o/n.

2.6.2.4 Washing of filter and autoradiography

Hybond filters were washed thee times for 30 min each in wash A (0.5 x SSC; 0.1 % SDS), at 65 °C. Filters were then blotted dry, wrapped in Saran wrap and placed against autoradiography sensitive film, in a light tight cassette containing an intensifying screen. After an appropriate exposure time of 2 - 5 hr or o/n, films were submersed, in the dark, in developer (Polycon™) for 5 min, and then fixative (Amfix™) for 5 min. Probe hybridisation, visible as exposed areas of film, was then orientated to individual clones, which were then be grown in
bacterial media. Clones were verified by small scale DNA extraction (2.3.1) and diagnostic restriction enzyme analysis (2.4.3).

2.7 Plant growth conditions

2.7.1 Seed germination

All harvested seeds were collected into individual 9 cm petri dishes, on top of a piece of filter paper to keep them dry. For germination, seeds were placed onto moist, sieved compost in 9 cm petri dishes, and incubated at 4 °C for 5 days to break dormancy. Petri dishes were then transferred to the green house, and once the cotyledons had emerged, the petri dish lids were removed. After the first true leaves had developed, seedlings were pricked out and planted in compartmented trays containing a compost/sand mix (2.7.2).

2.7.2 Compost, lighting and temperature

*A. thaliana* were grown under 24 hr lighting (400 W Sodium bulb), at 18 °C in a mixture of 0.75 % compost (Petersfield product) and 25 % silversand. *N. tabacum* plants, also under continuous lighting (65 W white light strip lights), were grown in 100 % compost at 25 °C.

2.8 Plant transformation

*A. tumefaciens* mediated transformation was used to generate transgenic tobacco and *A. thaliana* plants in tissue culture.

2.8.1 *N. tabacum* leaf disk transformation

As described by Draper et al., 1988.

2.8.1.1 *N. tabacum* tissue culture media

<table>
<thead>
<tr>
<th>MS0</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murashige &amp; Skoog basal media</td>
<td>4.4</td>
</tr>
<tr>
<td>sucrose</td>
<td>30</td>
</tr>
<tr>
<td>agarose</td>
<td>0.85</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MSD4X2</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSO</td>
<td>as above</td>
</tr>
<tr>
<td>10 mg/ml 6-BAP</td>
<td>100μl</td>
</tr>
<tr>
<td>10 mg/ml NAA</td>
<td>10 μl</td>
</tr>
</tbody>
</table>
2.8.1.2 *N. tabacum* transformation protocol

Medium sized, wild type SR1 tobacco leaves were surface sterilised in 10 % Domestos, for 15 min and washed three times in dH2O. 0.5 cm² leaf pieces (avoiding the mid-rib), were submersed and incubated for 20 - 30 min at RT, in 2 X YT containing appropriate transformed *A. tumefaciens* strain to a final OD of 0.1 (Abs600). Leaf disks were transferred to MSD4X2 plates, dorsal side up at a density of 20 - 25 per plate and incubated for 48 hr, under 12 hr illumination. Leaf disks were then transferred to MSD4X2 plates, 5 - 6 disks per plate, containing kanamycin (100 μg/ml) and augmentin (400 μg/ml) or cefotaxime (200 μg/ml). Plates were incubated at 25 °C, under 12 hr lighting, until leaf material produced callus and then shoots. Shoots were put on to MS0, containing kanamycin, 100 μg/ml and Augmentin, 200 μg/ml. Once rooted plants were put into compost and kept in a translucent plastic bag for 2 days, to retain the humidity.

2.8.2 *A. thaliana* root transformation

2.8.2.1 *A. thaliana* tissue culture media

Silver thiosulphate stock solution (1.25 mg/ml):-

Dropwise a solution of silver nitrate (2.5 mg/ml), was added to sodium thiosulphate pentahydrate (14.6 mg/ml).

Germination medium (GM)  

<table>
<thead>
<tr>
<th>Component</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 X MS</td>
<td>2.22</td>
</tr>
<tr>
<td>sucrose</td>
<td>10</td>
</tr>
<tr>
<td>MES</td>
<td>0.5</td>
</tr>
<tr>
<td>Difco bacto agar</td>
<td>8</td>
</tr>
</tbody>
</table>

media adjusted to pH5.8 using KOH

media was autoclaved and filter sterilised silver thiosulphate was added to a final concentration of 5 mg/L.

Callus-inducing medium (CIM)  

<table>
<thead>
<tr>
<th>Component</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamborg's B5</td>
<td>3.2</td>
</tr>
<tr>
<td>glucose</td>
<td>20</td>
</tr>
<tr>
<td>MES</td>
<td>0.5</td>
</tr>
<tr>
<td>Difco bacto agar</td>
<td>8</td>
</tr>
</tbody>
</table>

media adjusted to pH5.8 using KOH
media was autoclaved and silver thiosulphate (5 mg/L), 2,4-D (0.5 mg/L) and kinetin (0.05 mg/L) were added.

<table>
<thead>
<tr>
<th>Shoot-inducing medium (SIM)</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamborg's B5</td>
<td>3.2</td>
</tr>
<tr>
<td>glucose</td>
<td>20</td>
</tr>
<tr>
<td>MES</td>
<td>0.5</td>
</tr>
<tr>
<td>Difco bacto agar</td>
<td>8</td>
</tr>
</tbody>
</table>

media adjusted to pH5.8 using KOH
media was autoclaved and vancomycin (850 mg/L), kanamycin (35 mg/L), 2-iP (5 mg/L) and IAA (0.15 mg/L) were added.

<table>
<thead>
<tr>
<th>Shoot overlay medium (SOM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Made as SIM, but replacing the Difco bacto agar with 8 g/L low melting point agarose.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Shoot elongation medium (SEM)</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 X MS</td>
<td>2.22</td>
</tr>
<tr>
<td>sucrose</td>
<td>10</td>
</tr>
<tr>
<td>MES</td>
<td>0.5</td>
</tr>
<tr>
<td>Difco bacto agar</td>
<td>8</td>
</tr>
</tbody>
</table>

media adjusted to pH5.8 using KOH, and autoclaved.

<table>
<thead>
<tr>
<th>A. tumefaciens culture dilution medium</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamborg's B5</td>
<td>3.2</td>
</tr>
<tr>
<td>glucose</td>
<td>20</td>
</tr>
<tr>
<td>MES</td>
<td>0.5</td>
</tr>
<tr>
<td>Difco bacto agar</td>
<td>8</td>
</tr>
</tbody>
</table>

media adjusted to pH5.8 using KOH, and autoclaved.

**2.8.2.2 A. thaliana transformation protocol**

As described by Clarke et al., 1992. Wild type A. thaliana seeds (C24, Landsberg erecta or Columbia) were sterilised in 10% Domestos for 13 min. Seeds were rinsed 5 times in dH₂O, and spread on GM agar plates, at approximately 40 seeds per 9 cm petri dish. Plates were sealed with Micropore, 3M.
tape and incubated at 4 °C, for 5 days to break seed dormancy. Plates were then incubated for 3 - 4 weeks in a standard light regime for cultures (temperature: 20 - 22 °C), to allow root growth as a source of explant tissue. Once the seedlings had produced abundant (but not yet greening) roots, the whole seedlings were removed from the agar using forceps, and intact root systems were placed on agar plates containing CIM. CIM plates were incubated at 20 - 22 °C for 3 days. Roots were transferred to a sterile petri dish and cut into 0.5 cm lengths. Root explants were incubated at RT, for 2 min, with a 48 hr grown Agrobacterium culture, which had been diluted to a final OD of 0.1 (Ab 600). Roots were washed through a sieve to drain off the bacterial suspension, and blotted on sterile filter paper to remove excess liquid. Roots were placed back on CIM plates and incubated, as before, for 48 - 72 hr. Roots were washed using a sieve, with Agrobacterium culture dilution medium to remove excess bacterial growth, and blotted dry. Root explants were suspended in 10 ml of molten-SOM and overlaid on to 35 ml of SIM, ensuring the root material was evenly dispersed. After approximately 3 weeks, green (putatively transformed) calli develop from the root explants, and regenerate leaves over a period of several weeks. Expanded leaves were transferred into 60 ml plastic pots, containing SEM. Once shoots appeared, these were cut at their base and transferred into glass culture tubes, sealed with cotton wool bungs. Under these reduced humidity conditions, approximately 80% of the transformed shoots, flowered, rooted and set seed. Small scale DNA extraction (2.9.1) and PCR (2.9.2) was used to verify the presence of the transgene, and southern analysis (2.9.3), and plating of T3 seed on GM medium (containing 35 μg/ml kanamycin) were used to verify the T-DNA copy number.

2.9 Preparation and manipulation of plant DNA and RNA

2.9.1.1 Small scale preparation of plant genomic DNA

As described by Edwards et al., 1991. 1.5 ml microcentrifuge tubes were closed around fresh leaf lamina. Gathered plant material was stored on dry ice until extraction. The tissue was ground for 15 secs with a disposable grinder. 300 μl of DNA extraction buffer (200 mM Tris-HCl pH 7.5; 250 mM NaCl; 25 mM EDTA; 0.5 % SDS) was added and tissue was ground for a further 15 secs, before vortexing for 5 secs. Samples were stored at RT until all samples were ground. Extracts were spun for 1 min, and 200 μl of S/N was removed to a fresh microcentrifuge tube. 200 μl of isopropanol was added to the S/N and the tube was mixed and allowed to
precipitate at RT for 2 min. Precipitated DNA was spun down for 5 min at 14 000 g. Pellets were vacuum dried and resuspended in 50 µl. 2 µl samples were used per PCR reaction.

2.9.1.2 Preparation of plant genomic DNA for CAPS mapping

As described by Konieczny and Ausubel (1993), based on a method described by Dellaporta et al. (1983). One to three Arabidopsis leaves were placed in a 1.5 ml microcentrifuge tube, dipped in liquid nitrogen, and ground to a powder. 500 µl of extraction buffer, followed by 35 µl of 20 % SDS was added to the microcentrifuge tube and vortexed. The samples were incubated at 65 °C for 10 min and then 130 µl of 5 M KoAC was added. The samples were incubated at zero °C for 5 min and spun at 14 000 g for 10 min. The S/N was transferred to a new 1.5 ml microcentrifuge tube and 640 µl isopropanol and 60 µl 3 M NaOAc was added, samples were mixed and incubated at -20 °C for 10 min. Precipitated DNA was spun down for 15 min at 14 000 g, and redissolved in 200 µl of 50 mM Tris pH 8.0, 10 mM EDTA. Samples were spun at 14 000 g for 5 min to remove insoluble material, and the S/N was removed to a new microfuge tube and 440 µl of absolute ethanol and 20 µl of 3 M NaOAc was added. Samples were incubated at -20 °C, for 10 min, then DNA was spun down at 14 000 g for 5 min, and the pellet was washed with 70 % ethanol. Pellets were dried and dissolved in 50 µl dH2O.

2.9.2 PCR reactions

Oligonucleotide primers were synthesised on request from the Biochemistry Department. A 400 µl aliquot was ethanol precipitated (2.4.4) and resuspended in 400 µl. 5 µl of primer was diluted to 1 ml, and measured at an Ab260, primers were resuspended to a concentration of 20 µg/ml (OD of 1, at Ab260 = 20 µg/ml for single stranded oligonucleotide primers). PCR reactions were carried out in 50 µl volumes, in 0.5 ml microcentrifuge tubes, containing 2 µl of genomic DNA (2.9.1) or 1µl of DNA for CAPS analysis (2.9.1.2), 5 µl of 10 X PCR buffer (Gibco-BRL), 1 µl dNTPS (10 mM of each deoxynucleotide), 2.5 µl of each primer (20 µg/ml), 1.5 µl of MgCl2 (50 mM) and 0.25 µl of Taq polymerase. Samples were covered with 50 µl of mineral oil to prevent evaporation. 25 -35 PCR cycles were carried out in Perkin-Elmer DNA thermocycler. 1 cycle consisting of 94 °C (40 secs) denaturing
temperature, 50 - 60 °C (1 min) primer annealing temperature, depending on primer deoxynucleotide composition and length, and 72 °C (1 - 2.5 min) extension temperature. For CAPS PCR reactions, 30 cycles were carried out using a primer annealing temperature of 60 °C and an extension time of 1.5 min. All reactions were initiated with a "hot start" of 3 min at 94 °C and ended in 2 min at 72 °C. 25 μl from each reaction was then run on an agarose gel (2.4.1), for visual analysis (2.4.2). The Map Pairs™ primers were supplied by Research Genetics Inc. (Canada).

2.9.3 Southern analysis

As described by Southern, 1975. 5 - 10 μg of plant genomic DNA, digested with a single restriction enzyme, such as BamH1 or EcoR1, was run on a 0.8 % agarose gel (2.4.1), until the bromophenol dye front was close to the bottom of the gel. Gels were photographed on a transilluminator, with a ruler alongside, so that once autoradiographs were developed, visible bands could be sized. Gels were incubated at RT, for 10 min in depurinating solution (0.25 M HCl), 0.5 hr in denaturing solution (0.5 M NaOH; 1.5 M NaCl) and then 0.5 hr in neutralising solution (3 M NaCl; 0.5 M Tris-HCl, pH 7.4). Genomic DNA was transferred, in position, to a nylon membrane (Hybond-N, Amersham), via capillary action of 20 x SSC (88.2 g/L Na citrate; 175.3 g/L NaCl). Membranes were dried and DNA was autocrosslinked (DNA Stratalinker - Stratagene). Generation of probe stocks, prehybridisation of filters and hybridisation of DNA probes were carried out as described previously (2.6.2.2 - 2.6.2.4).

2.9.4 Small scale preparation of plant RNA

As described by Guerineau et al., 1991. Plant material was frozen and ground in TLES buffer (50 mM Tris-HCl, pH 9, 150 mM LiCl, 5 mM EDTA, 5 % SDS) and 2 phenol chloroform extractions were performed on the lysate. Total nucleic acids were precipitated with ethanol, then centrifuged and dissolved in 50 μl dH2O. One volume of 4 M LiCl was added and RNA was precipitated on ice for 30 min. After centrifugation, RNA pellets were washed with 70 % ethanol, dried, dissolved in 20 μl of dH2O and either processed for analysis or stored at - 80 °C.
2.9.5 cDNA synthesis

7 µl of RNA sample was incubated with 5 µl of 5 x reverse transcriptase buffer; 2.5 µl of 0.1 M DTT; 2.5 µl of 10 mM dNTPs; 1 µl of 50 ng/µl oligo dT primer and 1 µl of RNasin for 10 min at RT. 1 µl of reverse transcriptase (superscript) was added, and the sample was incubated at 37 °C for 1 hr. The resulting cDNA sample was then diluted to 100 µl with dH2O, and 1 µl was used in 35 PCR cycles, carried out as described above (2.9.2), with a 1.5 min time span to reduce from the denaturing temperature to an annealing temperature of 62 °C, held for 30 secs, and an extension time of 1.5 min.

2.9.6 Double stranded DNA sequencing
2.9.6.1 Denaturing plasmid DNA

DNA plasmid preparations (2.3.1), were denatured to give single stranded templates. 18 µl of plasmid DNA was denatured by adding 2 µl of 2 M NaOH and 2 µl of 2 mM EDTA, for 5 min at RT. Samples were neutralised with 2 µl of 2 M NH4Ac (pH4.6), mixed quickly and 60 µl of absolute ethanol was added. Samples were incubated for 5 min on ice, and spun at 14 000 g for 8 min to precipitate single stranded DNA templates. DNA templates were washed with 70 % ethanol, dried and resuspended in 7 µl of dH2O.

2.9.6.2 Annealling sequencing primers

DNA fragments contained in pUC vectors was sequenced using either the universal primer (UP) site to sequence the sense strand, or the reverse primer (RP) to sequence the antisense strand of the DNA insert. To the single stranded templates, obtained from above (2.9.6.1), 1 µl of UP or RP and 2 µl of 5 x sequencing buffer was added. Samples were incubated in a beaker of water at 65 °C for 5 min, and then allowed to cool at RT until the water temperature in the beaker dropped to 35 °C or below. At this stage the samples can either be frozen at -20 °C, or processed directly in sequencing reactions (2.9.6.3).
2.9.6.3 Sequencing reactions

Four microcentrifuge tubes, containing 2.5 µl of one of each nucleotide, dTTP, dCTP, dGTP and dATP, were set up per sequencing reaction. In a separate microcentrifuge tube, labelling reaction comprising 1 µl of 0.1 mM DTT, 2 µl of diluted labelling mix (diluted to 1 in 5 µl), 2 µl of diluted sequenase enzyme (diluted 1 in 8 µl) and 1 µl of α35S dATP (DuPont) were added together, mixed and 5.5 µl was added to the 7 µl of single stranded template prepared for sequencing (2.9.6.2). At time point zero, 3.5 µl of template/labelling mix was added in succession to the four microcentrifuge tubes, containing the four different nucleotides, at 37 °C, and at a time point of 5 min, 4 µl of sequencing stop solution was added to stop the reactions. The four sequencing reactions were incubated in a boiling water bath, just below boiling point, for 2 min and transferred to ice. Samples could then either be frozen at -20 °C, or loaded directly on to a sequencing gel (2.9.6.4).

2.9.6.4 Sequencing gel and autoradiography

Sequencing reactions were run on a 6 % acrylamide/urea gel, using sequencing equipment from Bio-Rad. The front and back gel plates and the spacers were washed with water and wiped with IMS. The front gel plate was then evenly coated with Repelcoat™, to prevent the gel sticking to the front plate, and wiped with IMS to remove any HCl produced. Spacers were inserted between the gel plates and clipped together. To seal the bottom of the gel, 2 strips of 3MM were placed on the sponge of the casting tray, and 10 ml of acrylamide/urea containing 50 µl of TEMED and 50 µl of 25 % amps were poured on top of the 3MM. The gel was gently rested on the 3MM, and acrylamide/urea mix was allowed to penetrate and seal the bottom of the gel. Once set, the main gel consisting of 50 ml acrylamide/urea with 50 µl TEMED and 100 µl of amps, freshly added, was syringed from the top, between the two gel plates. The flat side of a sequencing gel comb was inserted into the top of the unset gel mix, clamped to ensure a tight seal, and allowed to set. The gel was then inserted vertically in the bottom electrode tray containing 0.5 x TBE running buffer, which was also poured down the back plate behind the gel. The comb was removed from the gel, cleaned and inserted back into the gel the other way up, so the comb teeth point down, and become inserted just into the gel, to make the wells to load the sequencing reactions (2.9.6.3). Using a syringe and needle containing 0.5 x TBE, the wells were cleaned to expel any

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urea and 2.5 μl of each sequencing reaction, were loaded into the wells in the order T, C, G, A. The top electrodes were connected and the gel was run at 2000 V (22 mA), until the first dye front reaches the bottom of the gel. Carefully the side clips and front gel plate were removed and the gel was bathed in 10% acetic acid/10% methanol solution for 20 min. A large piece of 3MM was then laid on top of the gel, which was then lifted from the back plate. Cling film was laid over the gel, which was cut down to size, to remove excess acrylamide where no reactions had been run. The gel was then dried in a vacuum heater for 30 min. Once dry the cling film was removed, and in the dark a piece of autoradiography film was placed over the gel. After 24 hr, the autorad was developed in the automatic developer, and the nucleotide sequence of the insert was evaluated, from the banding patterns of the four separate nucleotide lanes.

2.10 Analysis of transformants with reporter gene constructs

In order to evaluate the temporal and spatial patterns of transgene expression, selected plant material was ground in phosphate extraction buffer. GUS and LUC reporter gene activities were then either qualitatively or quantitatively assayed and all were standardised by expressing the results as a function of protein concentration and shown graphically with bud/anther length against GUS/LUC activity. Floral buds were sized to ascertain developmental stage, by measuring the bud length in Arabidopsis (using a light microscope and a graticule), and the anther length in tobacco (using a ruler with mm markings). 1 - 5 Arabidopsis buds or 0.5 - 2 tobacco anthers were ground in 200 μl of extraction buffer, containing alumina to aid thorough grinding. Samples were then spun for 1 min at 14 000 g to remove the debris and alumina, the S/N was removed to a fresh microcentrifuge tube and stored on ice before assaying.

2.10.1 Histochemical detection of the GUS protein

Plant material was incubated in 10 μg/ml X-GLUC and protein fixer, at 37 °C for 2 hr, after which time GUS activity was visualised in situ, by a blue colouration.

2.10.2 Fluorimetric analysis of the GUS gene

Plant material was ground in extraction buffer (10 mM EDTA, 10 mM β-Me, 0.1% triton-X100, 50 mM NaPO₄ buffer pH7). Individual samples were diluted to contain an equivalent quantity of protein (2.10.4), and 100 μl was incubated at 37 °C. 10
mM MUG was added at time point 0, and after 5, 10, 20, 60 and 90 min intervals, 20 μl samples were removed and pipetted into a microtitre plate well containing 180 μl of 2 M NaCO₃, to stop the reaction. Once all samples were collected GUS activities were measured as a function of fluorescence, using a fluorimeter. The concentration of MU produced was then calculated by comparisons with known standards and data was presented as nmoles MU produced per min per mg protein.

2.10.3 Luminescence analysis of the LUC gene

Plant material was ground in extraction buffer (100 mM KPO₄ pH 7.8, 1 mM DTT) in the present of alumina. Extracts were spun for 1 min and the S/N stored on ice until assayed. 10 - 20 μl samples were incubated with ATP (10 mM ATP, 20 mM MgCl₂, 50 mM HEPES) and luciferin (0.05 mM luciferin, 100 mM KPO₄ pH 7.8, 1 mM DTT). Luciferase activity, measured as luminescence, was counted for 10 secs and recorded as units. Protein concentrations (2.4.10) were calculated for each of the samples and LUC activity was expressed as units per μg protein.

2.10.4 Measurement of protein concentrations

Protein standards between 0.1 and 0.7 μg of protein were made in phosphate buffer, using β-globulin. 10 μl of each sample was added to 790 μl of dH₂O, to which 200 μl of Bradfords reagent was added (Bio-Rad; Bradford, 1976). Samples were measured in a Hewlett-Packard spectrophotometer at Abs 595. Absorbance was plotted against concentration, the gradient of the resulting straight line was 28.9. Therefore the Abs 595 x 28.9 gave the μg protein present for each sample assayed.

2.11 Microscopy techniques

2.11.1 Light microscopy

To enable a comparison of sporangium histology, anther transverse sections were prepared from each of the A. thaliana, GUS negative (gne) mutants isolated, and from the wild type, C24. Sections were observed using light microscopy and compared to wild type sporangium development.
2.11.1.1 Tissue fixation, dehydration and resin embedding

Day 1 - Tissue fixation. Mutant and wild type *A. thaliana* floral buds were harvested and sized. A healthy whorl of buds was removed from the plant using watch maker forceps, individual buds were separated from each other using a scalpel blade and placed on a microscope slide. With the use of a graticule, placed beneath one of the light microscope eyepieces, bud lengths were measured to the nearest 1/100th of a mm. Whorls of buds were examined and collected in this way, from one individual plant, for each gne mutant and WT, until a distribution of buds, ranging from 0.3 to 1.0 mm in length were gathered. Individual buds were placed in 1.5 ml microcentrifuge tubes, containing 50 μl of fixative (5 % gluteraldehyde, 50 mM phosphate buffer, pH 7). Open microcentrifuge tubes were vacuum infiltrated for 30 min, to allow a better penetration of fixative and samples were then stored o/n at RT.

Day 2 - Ethanol dehydration. Buds were dehydrated by sequential incubation through an ethanol series of increasing concentration. Using a pasteur pipette (whose end had been heated and extruded, to create an opening smaller than the smallest bud, to prevent samples being lost) fixative was removed from the microcentrifuge tube and sequentially replaced with 100 μl of 10 %, 30 %, 50 %, 70 %, 90 % and 100 % ethanol. Dehydration was carried out on ice, and samples were incubated for 1 hr between dehydration steps. Buds were incubated twice more in fresh 100 % ethanol, for 30 min each time and samples were then left in 100 % ethanol, at 4 °C, o/n.

Day 3 - Exchange into propylene oxide. 100 μl of propylene oxide was added to each tube, already containing 100 μl of absolute ethanol. Samples were incubated at 4 °C for at least 5 hr. Using a pasteur pipette propylene oxide/ethanol was removed and replaced with 200 μl of propylene oxide. Samples were incubated o/n at 4 °C.

Day 4 - Exchange into Araldite resin. Samples were incubated for 30 min, at RT, in 2 exchanges of 100 μl of propylene oxide. 100 μl of Araldite resin was added to the sample in 100 μl propylene oxide and incubated at RT for 2 hr.

Araldite resin:

- Araldite: 5 ml
- DDSA: 5.5 ml
- dibutylphthalate: 250 μl (plasticiser)
- BDMA: 180 μl (catalyst)
Araldite and DDSA were heated to 60 °C, mixed thoroughly in a warm universal, allowed to cool to RT and then plasticiser and catalyst were added. Reagents were obtained from Agar Scientific.

Propylene oxide/Araldite was removed using a pasteur pipette and replaced with 100 µl of Araldite, samples were vacuum infiltrated for 15 min and left o/n at RT.

Day 5 - Araldite embedded samples set in microcapsules. Araldite embedded buds were removed from microcentrifuge tubes using a small plastic rod and placed at the bottom of microcapsules containing Araldite resin. Samples were vacuum infiltrated for 30 min and then incubated at 37 °C for 3 hr. Samples were then incubated at 60 °C for 48 hr, to allow the resin to set solid.

2.11.1.2 Sample orientation, sectioning and staining

Sample orientation. Microcapsules were cut away from set araldite blocks. Under a binocular, dissecting microscope the tips of the Araldite blocks, containing the embedded bud, was cut away using a scalpel blade. Araldite tips were orientated so that the bud was perpendicular to the remaining block, and using Araldite glue was stuck back on. The glue was allowed to set at RT for at least 24 hr, before sectioning.

Sectioning. 5 µm bud transverse sections were prepared using a Huxley Ultramicrotome. Using a scalpel blade, block faces were prepared: excess Araldite above and around the bud was removed, leaving the block face shaped as a parallelogram, containing the bud. Blocks were then fixed in place on the microtome and using a glass knife, which advanced towards the block, 5 µm sections were cut. Plastic boats fixed to the glass knives, contained dH₂O, which caught the sections and prevented them from damage, as they were cut from the block face.

Staining of sections. Sections were removed from the boats, using a special metal loop, and placed on a microscope slide. Slides were heated on a hot plate (80 °C) until all the dH₂O had evaporated. A solution of filtered, 1 % Toluidine blue was placed on top of, and covering, all the sections. The Toluidine blue stain was heated on the sections, on the hot plate, for approximately 30 secs (ensuring that none of the sections dried out). Excess blue dye was washed from the sections using dH₂O and sections were dried on the hot plate, before viewing using a light microscope.
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Staining of sections. Sections were removed from the boats, using a special metal loop, and placed on a microscope slide. Slides were heated on a hot plate (80 °C) until all the dH2O had evaporated. A solution of filtered, 1 % Toluidine blue was placed on top of, and covering, all the sections. The Toluidine blue stain was heated on the sections, on the hot plate, for approximately 30 secs (ensuring that none of the sections dried out). Excess blue dye was washed from the sections using dH2O and sections were dried on the hot plate, before viewing using a light microscope.
2.11.2 Scanning electron microscopy

Samples prepared for examination by scanning electron microscopy (S.E.M.), were fixed in glutaraldehyde and transferred through an ethanol gradient as described for day 1 and day 2 samples prepared for light microscopy (2.11.1.1). Additionally samples were then transferred through from 100 % ethanol to 100 % acetone, again through a graded series of acetone steps, as for the ethanol treatments. Samples were then dried in a critical point dryer and sputter coated (2.11.2.1).

2.11.2.1 Critical point drying, sputter coating and visualisation of specimens

Samples were placed, submerged in acetone in the chamber of a critical point drier (Blazers), and cooled to 7 - 8 °C. While being stirred the acetone was replaced by liquid CO\textsubscript{2}, which was flushed through the chamber three times. The chamber was heated until the critical point was reached (Approximately 31.5 °C and 73.8 bar), turning the liquid CO\textsubscript{2} into a gas, which was expelled from the chamber. The samples were removed and transferred to the sputter coater, where samples were coated with gold to an approximate thickness between 150 to 300 Å, to allow visualisation of the samples with the electron beam. Samples were then examined using a Cambridge S.100 scanning electron microscope.

2.11.3 Transmission electron microscopy

Floral buds for examination by transmission electron microscopy (T.E.M.) were sized, as for light microscopy, and embedded in Spurr resin (courtesy of Stephan Hymen, E.M. laboratory).

2.11.3.1 Sectioning, staining and visualisation of sections

Anther transverse sections of "silver" thickness (approximately 80 nm), were sectioned using glass knives on a Huxley ultramicrotome. Sections were expanded, to removed any wrinkles, by passing a cotton bud soaked in chloroform over the top of the sections, which were then picked up on to cleaned 3.05 mm copper grids (Agar Scientific) using reversible forceps, and allowed to dry under a normal lamp. Samples were then stained using a series of pre-filtered solutions, to allow for a contrast within the different specimen tissue types. First grids were immersed, face up, in droplets of saturated uranyl acetate (4 %), mixed 50/50 with
distilled water, for 20 min on a wax surface, held in a chamber with methanol at the bottom. Grids were removed and dipped into two washes of 50% methanol, followed by two washes in dH₂O. Grids were then immersed face down in droplets of lead citrate (0.2 g lead citrate/10 ml dH₂O with 0.1 ml of 10 M NaOH), for 3 min on a wax surface, with KOH pellets in the bottom of the chamber. Grids were quickly removed and dipped into 0.1 M KOH and washed three times in dH₂O. Processed grids were dried under a lamp, and then examined using a Jeol 100CX transmission electron microscope.

2.12 in situ hybridisation

2.12.1 Fixation and sectioning of buds and pronase E digestion

A whorl of buds from the apex of WT, gne1, gne2, gne3 and gne5, joined together by the inflorescence stem were completely immersed and fixed at RT in 4% paraformaldehyde in small specimen vials. After 24 hr, the paraformaldehyde was replaced by an equivalent volume of 0.5 M sucrose in PBS at 4°C, and incubated for a further 24 hr. Samples were exchanged into 100% ethanol and then 100% xylene, both through a graded series of exchanges. Floral inflorescences were vacuum embedded in semi-synthetic hot wax, and allowed to cool.

The block faces from each of the 5 samples were sectioned to produce 5 μm thick strips of wax ribbons, to produce serial sections from the tip to the base of each whorl. To visualise the wax sections using a light microscope, samples were placed on treated slides with DEPC treated water (0.1 % DEPC added to dH₂O, mixed and autoclaved) and heated on a hot plate, to stretch the samples allowing them to dry flat and stick on to the slides. Treated slides were made by washing microscope slides in decon, followed by distilled water, a quick dry and then dipped in 2% 3(triethoxysilyl)-propylamin in acetone, followed by 2 washes in acetone, then distilled water and finally dried at 60°C. Samples stored in this fashion were then stable at RT, indefinitely. Wax sections, through varying degrees of each whorl were examined for suitable samples which showed the fully differentiated microsporangium, and a clearly defined tapetal layer, prior to the disintegration of these cell types. 12 such wax sections placed on treated slides, were chosen from each mutant line and WT (only 10 were chosen for gne5 while 14 were chosen from WT), which were set aside for in situ hybridisation.

Prior to hybridisation slides were racked in special metal carriers and
washed through a series of coplin jars, at RT, comprising 2 successive xylene (Histoclear) incubations for 15 min each time, to de-wax the slides, followed by a 10 min wash in 100 % IMS and rehydration through 80, 60 and 30 % IMS and finally dH₂O for 1 min each. Slides were then rinsed in phosphate buffer, pH 7.4, and incubated at 37 °C in phosphate buffer, pH 7.5, containing 125 µg/ml pronase E for 10 min. Slides were then washed at RT in PBS/glycine, PBS and then 4 % paraformaldehyde in PBS for 10 min. Slides were rinsed in water and dehydrated through a series of graded ethanol steps into absolute ethanol and finally dried. Slides were then ready for pre-hybridisation (2.12.3).

2.12.2 Probe template preparation and ³⁵S labelling

Two tapetal-specific genes, A8 and A9, were chosen for in situ analysis on the WT and GUS negative mutants. Probes were constructed from pA8.1 (A8 in bluescript) and pWP67A (A9) plasmids, in sense and antisense orientations, representing negative and positive controls respectively. Mini DNA preparations (2.3.1) of pA8.1 and pWP67A, were carried out and the templates were linearised to completion to avoid the production of very long transcripts, which may incorporate a substantial fraction of the α³⁵S-UTP. In both plasmids, a suitable BamHI enzyme site (that gives no 3' overhangs, to eliminate extraneous templates which have been reported with templates that do contain 3' overhangs) lay downstream of the genes, suitable to allow transcription from the upstream T7 site to allow the production of the sense probes, used as a negative control. While Hind III sites upstream of both genes, were suitable cleavage sites to allow T3 transcription and production of the antisense probes.

DNA minipreparations of pA8.1 and pWP67A were digested with Hind III or BamHI, in a large total volume of 150 µl, comprising 5 µl of plasmid DNA; 15 µl of enzyme; 15 µl of react2 for Hind III digests or react3 for BamHI digests; and 115 µl dH₂O. After the digests had warmed to 37 °C, spermidine was added to 4 mM, and digests were incubated for a further 2 hr. Digests were extracted twice with an equal volume of 25:24:1 v/v phenol:chloroform:isoamyl alcohol and twice with 24:1 v/v chloroform:isoamyl alcohol. DNA was ethanol precipitated, washed twice with 70 % ethanol, dried and resuspended in dH₂O at 1 µg/ml. Digested plasmids were run on an agarose gel alongside uncut plasmid, to check templates were fully linearised.
For template transcription 5 µl of 5 x transcription buffer (supplied with the polymerase, Promega); 2 µl of 100 mM DTT; 4 µl of 10 mM dNTPs (dUTP, dATP, dCTP, dGTP); 10 µl of 12.5 mCi/ml ^35S-UTP (DuPont); 1 µl of DNA template and 1 µl of T3 (Hind III digests), or T7 (BamHI digests) RNA polymerase were added in a microfuge tube and incubated at 37 °C, for 35 min, after which time a further 1 µl of polymerase was added and incubated for an additional 40 min. DNA templates were removed by adding 1 µl of 1 M DTT; 2 µl of 20 mg/ml yeast RNA; 2 µl of RNasin and 0.5 µl of DNasel (Promega), samples were incubated for a maximum of 10 min at 37 °C. Protein was removed from the samples by adding 374 µl of 10 mM DTT. 400 µl of phenol/chloroform/isoamyl alcohol was then added to each sample, vortexed and spun for 2 min at 14 000 g, the upper phase was removed and precipitated with 40 µl 3 M NaAc and 1 ml ethanol. Samples were stored on dry ice for at least 1 hr and then spun at 14 000 g, for 20 min, pellets were vacuum dried for 15 min and resuspended in 100 µl of 10 mM DTT. Probes were hydrolysed to generate smaller fragments to ease penetration of tissue. 100 µl of hydrolysis buffer (80 mM sodium hydrogen carbonate; 120 mM sodium carbonate; 20 mM β-Me) was added to each probe and incubated for "T" min, where:-

\[ T = \frac{L_0 - L_f}{0.11} \]

and Lo is the original fragment length (approximately 0.45 kb for A8 and 0.9 kb for A9) and Lf is the desired fragment length (0.1 - 0.2 kb). After T min 200 µl of hydrolysis stop buffer was added (1 µl of 20 mg/ml yeast RNA; 40 µl 3 M NaAc and 1 ml ethanol). Samples were then precipitated on dry ice for at least 1 hr, spun for 15 min at 14 000 g, dried and resuspended in 10 mm DTT to a final concentration of 5 ng/µl. Hydrolysed probes were stored at -20 °C, prior to hybridisation with tissue samples (2.12.4).

2.12.3 Pre-hybridisation of samples

Sample slides were prehybridised in 1 L of pre-hybridisation buffer (0.7 g yeast RNA; 10 ml of 1 M DTT; 100 ml of 10 x salt mix (3 M NaCl; 100 mM sodium phosphate buffer, pH 6.8; 100 mM Tris-HCl, pH 7.5; 50 mM EDTA, pH 8; 0.2 % w/v BSA, Ficoll 400 and PVP); 500 ml of formamide; 390 ml of sterile dH2O), for 1 hr at 55 °C, dipped in 2 absolute ethanol washes, dried and stored in the fridge at
2.12.4 Hybridisation of samples with labelled probe

Each samples was etched with a diamond pencil, to indicate the probe used and specimen code, while the underside was marked with a marker pen to indicate the position of the sample. A paper towel was placed in the bottom of the hybridisation chamber, and 50 ml of formamide was poured on top. 1 ml of 50 % dextran sulphate was heated to 80 °C, to make it more tractable, then 4 ml of hybridisation buffer was mixed, comprising 400 μl of 10 x salts mix (2.12.3); 2 ml of formamide; 140 μl of yeast RNA; 40 μl of 1 M DTT; 620 μl of dH₂O; and 800 μl of 50 % dextran sulphate. Each of the four probes, A8 and A9 sense and antisense, were then individually added to 850 μl of hybridisation buffer, heated to 80 °C for 1 min and mixed thoroughly.

Using a calibrated pipette, 35 μl of hybridisation solution containing the appropriate probe was pipetted on to the labelled slides, and coverslips were gently lowered on top using forceps. For gne1, gne2, gne3 and WT, 5 slides were hybridised per antisense probe, while only 4 slides were hybridised for gne4. For gne1, gne2, gne3 and gne4, 1 slide was hybridised per sense probe, while 2 slides were hybridised for WT, giving a total of 60 slides. Slides were held on racks in the hybridisation chamber, which was sealed and incubated at 50 °C o/n.

2.12.5 Post hybridisation washing buffers

Each slide was removed from the hybridisation chamber, and immersed in 400 ml of formamide washing buffer (1 x salts mix; 50 % formamide) in a beaker. Using the blunt end of a pair of forceps the coverslip was dislodged from the top of each slide and allowed to fall to the bottom of the beaker. Slides were racked and stored in a sandwich box containing fresh formamide washing buffer, and washed in a 50 °C, shaking water bath for 1 hr. Fresh formamide buffer was added to the slides twice more with two further 1 hr incubations. Slides were then immersed into 7 changes of approximately 650 ml RNase buffer (5 L = 146 g NaCl; 50 ml of 1 M Tris-HCl, pH 7.5; 10 ml of 0.5 M EDTA, pH 8) at 37 °C, and in the last wash 1 ml of 75 mg/ml RNase A, which had been boiled for 1 min, was added to the RNase buffer at 37 °C and incubated with gentle shaking for 1 hr. Slides were transferred to 1 L of 2 x SSC (100 ml of 20 x SSC; 900 ml dH₂O) at 65 °C for 30 min with
gentle shaking, after which time the buffer was changed for fresh 2 x SSC buffer. Slides were then dehydrated through graded alcohols and dried, ready for autoradiography and development (2.12.5).

2.12.6 Autoradiography, developing and staining

For autoradiography, 10 g of Ilford K5 emulsion and 6 ml of water/glycerol mix were melted and mixed by inversion in the dark at 45 °C for 1 hr. The K5/water/glycerol mix was placed in a small beaker in the dark, and a blank test slide was 3/4 dipped into the solution and held up to the safe light to ensure that an even coating of emulsion was achieved. Hybridised slides were coated in emulsion in the same way, and excess emulsion was wiped from the back of each slide. Coated slides were stood at 30 degrees to the vertical in perspex racks with the section uppermost, and allowed to dry for 2 hr. Slides were racked into light tight slide boxes, sealed with plastic tape, wrapped in black polythene and stored at 4 °C.

After 21 days the slides were unwrapped and immersed in a beaker of D19 developer, 160 g/L, (Kodak), for 2 - 4 min at 15 -17 °C. Slides were then immersed in 1 % acetic acid, for 1 min, also at 15 -17 °C and fixed in Kodak unifix (150 g/L) for 4 min, at 15 -17 °C. At this point the lights were switched on and the slides were immersed in water for 2 min. To stain the slides, they were fixed in 10 % neutral buffered formol-saline for 5 min, slides were immersed in Harris's Haematoxylin for 2 min and then transferred to 50 % IMS for 1 min. Slides were then immersed in acid alcohol to remove the background staining of emulsion, and again back into 50 % IMS before being differentiated in Scott's tap water substitute for 1 min. Samples were then sequentially dipped through 30, 60 and 70 % IMS for 1 min each time, washed twice in absolute ethanol for less than 2 min, cleared with Histoclear (Xylene), for less than 5 min and finally coverslips were mounted over the samples using DPX (Agar Scientific).

2.13 In vitro protein synthesis

In vitro protein synthesis of the pAF19 derived AG coding region, from a pGEM-42 clone (pAF23), was carried out utilising T7 RNA polymerase (Stratagene) and the wheat germ translation system (Promega). A small scale Wizard™ mini DNA preparation of pAF23 was prepared (2.3.1), digested and boiled to generate a
single stranded DNA template. 25 µl of the 50 µl pAF23, DNA preparation was digested with 6 µl EcoRI restriction endonuclease, situated downstream of the T7 polymerase primer site and the AG coding region, along with 6 µl of React3 made up to a total of 60 µl with DEPC treated dH2O (0.1 % DEPC added to dH2O, mixed and autoclaved), digest was incubated at 37 °C for, 2 hr and then run on a 0.8 % gel. The 3.5 kb linearised pAF23 plasmid was cut from the gel and recovered by genecleaning (2.4.6.2) into a final volume of 20 µl DEPC treated dH2O.

To transcribe the AG gene, 5 µl of 5 x transcription buffer; 1 µl of 0.75 M DTT; 4 µl of 10 mM dNTPs (dUTP, dATP, dCTP, dGTP); 1 µl of RNAsin; and 1 µl of T7 RNA polymerase were added to 13 µl of pAF23 genecleaned template in a 1.5 ml microfuge tube, and incubated for 37 °C for 30 min. 200 µl of DEPC-dH2O was added to the tube, which was extracted once with an equal volume of phenol/chloroform and the DNA and RNA was precipitated with 3 M NaAC (DEPC treated) and 2.5 volumes of absolute ethanol. The sample was placed at -80 °C for 15 min, pelleted by centrifugation at 14 000 g for 10 min and washed in 80 % ethanol (made with DEPC-dH2O). Sample was air dried for 30 min and resuspended in 20 µl of DEPC-dH2O. In the translation reaction (kit supplied by Promega), 12.5 µl of wheat germ extract; 0.5 µl of RNAsin; 2 µl of amino acids - methionine; 0.75 µl of KoAC; and 1.25 µl of 35S methionine were added to a 1.5 ml microcentrifuge tube (the wheat germ extract was handled carefully and not vortexed or centrifuged).

The AG RNA transcription product was heated for 10 min at 76 °C and then put on ice (to unfold the RNA allowing better translation) 8 µl of RNA was then added to the 17 µl of the translation reaction made above and incubated at 25 °C, for 1 hr. After which 8.3 µl of 3 x cracking buffer was added, and the sample was boiled for 3 min. Half of the reaction was loaded and run on to an 11 % SDS/acrylamide gel (2.14), along with sized protein markers. The gel was stained with Coomassie, and the movement of the protein markers from the top of the gel were noted, the gel was subsequently destained in 1 % TBS/tween, placed on to 3MM, covered with cling film and dried in a gel drier for 2 hr. The dried gel was put down on to autoradiography film and developed after 24 hr in the automatic developer.
2.14 Preparation and running of mini-protein gels

To prepare an 11% protein gel, 100 µl of 100 mg/ml amps and 12 µl of TEMED was added to 7.5 ml of running gel (5.5 ml of 30% acrylamide/0.8% bis solution; 5.6 ml of dH₂O; 3.75 ml of 1.5 M Tris-HCl, pH 8.8 and 0.15 ml of 10% SDS) which was poured between spaced plates held in a mini gel kit casting kit (Bio-Rad); the unset running gel was overlaid with 1 ml of saturated butan-2-ol, to exclude oxygen and allow setting. Once set, the butan-2-ol was poured off, and the top of the gel was dried with filter paper. 80 µl of amps and 10 µl TEMED was added to 5 ml of stacking gel (1.67 ml of 30% acrylamide/0.8% bis solution; 6.9 ml of dH₂O; 1.25 ml of 1.5 M Tris-HCl, pH 8.8 and 0.1 ml of 10% SDS), which was then poured on top of the running gel, into which a comb was inserted. Once set, gels were transferred into tanks (Bio-Rad), filled with 10 x TBS running buffer (3% Tris base; 14% glycine; 1% SDS). 3 x Cracking buffer (3.75 ml of 0.5 M Tris-HCl, pH 6.8; 1.5 ml β-Me; 3 ml glycerol; 0.6 g SDS; 0.04 g bromophenol blue) was added to protein samples to produce a 1 x solution, which was boiled for 1-3 min prior to loading on the gel. The wells of the gel were expelled of urea using a pipette just prior to loading. The gel was run at 150 volts, until the loaded samples had moved from the stacking gel into the running gel, and then the voltage was turned up to 200 volts until the bromophenol blue dye front had reached the bottom of the gel. The gel was then removed from the tank, and the front plate removed leaving the gel on the back plate which could be immersed into a container to visualise the protein bands using a 0.2% ponceau stain (in 3% trichloroacetic acid).
Chapter 3
Sense and antisense expression of the floral homeotic gene, *AGAMOUS*, under the control of two tapetal-specific promoters, A6 and A9
3.1 Introduction

AG is a homeotic gene involved in the regulation of floral organ specification in A. thaliana (1.1.1). The ag-1 A. thaliana mutant, generated by EMS mutagenesis maps to chromosome IV (Koomneef et al., 1980), and produces indeterminate flowers comprised of sepals and petals (Bowman et al., 1989). In ag-1 flowers the floral primordia of the first, second and third whorls develop in their WT positions, and the organs of the first two whorls develop normally as sepals and petals respectively, whereas the primordia of the third whorl develop into petals instead of stamens. However the primordial cells of the fourth whorl that normally develop into the gynoecium, take the fate of a new ag-1 floral primordium, producing sepal and petal primordia which in turn contains a new floral primordium at their centre, and so on resulting in a flower that may be composed of up to 70 floral organs. Thus ag-1 flowers comprise an outer whorl of four sepals, then ten petals and and inner flower comprised of variable numbers of sepals and petals, in addition to which the sepals of the internal flower develop as mosaics of sepals and petals (Bowman et al., 1989). These studies show that AG has a function in the specification of stamen and carpel identity and in the control of determinacy of the flower.

The isolation of a second allelic mutation in the AG gene, ag-2, generated by T-DNA insertional mutagenesis (Feldmann et al., 1989), enabled the cloning of the AG gene. Plant DNA sequence from the ag-2 mutant, flanking the T-DNA insertion site, was isolated by plasmid rescue and used to screen a WT A. thaliana cosmid library, one of the cosmids isolated, complemented the ag-2 phenotype, indicating this clone contained the AG gene (Yanofsky et al., 1990). A putative AG cDNA clone was also isolated and sequenced, and used to isolate sequence from the ag-1 mutant, comparison of the two allelic sequences showed a single bp change from G to A in the ag-1 mutant, confirming the AG gene sequence (Yanofsky et al., 1990). A search of the protein database revealed that the AG protein sequence, shared a region of sequence homology with transcription factors, such as SRF isolated from humans (Norman et al., 1988), MCM1 from yeast (Passmore et al., 1988) and DEFICIENS A (Sommer et al., 1990). This region of sequence, with which AG shares approximately 80 % homology, spans approximately 56 amino acids in length and contains a putative DNA binding and dimerisation domain, now termed the MADS box domain, after the initial description of this DNA binding domain in these four proteins MCM1, AG,
DEFICIENS and SRF (Schwarz-Sommer et al., 1990).

To determine the target DNA sequence bound by the AG protein, the AG MADS domain with an added antibody epitope tag was over expressed using the E. coli T7 expression system, and added in vitro, to a mixture of double stranded oligonucleotides with a random sequence of 40 nucleotides in the central region. Immunoprecipitation of the MADS domain and associated bound oligonucleotides, allowed a comparison of the DNA fragments bound by the AG MADS domain and revealed the consensus sequence 5'-TT(A/T/G)CC(A/T)6GG(A/T/C)AA-3' (Shiraishi et al., 1993). DNase I footprinting and methylation interference experiments confirmed this motif as the target of the AG MADS domain.

In situ hybridisation studies using antisense AG RNA probe, has shown how the pattern of AG expression changes in a mutant ap2 background as compared to WT floral development, and how later in development AG RNA is restricted to certain cell types (Drews et al., 1991; Bowman et al., 1991a). In WT floral primordia, AG RNA is first detected at a time when the sepal primordia first appear, at late stage 2 and early stage 3, but prior to the emergence of either the petal, stamen or carpel primordia (Drews et al., 1991). An RNA signal is detected only over a hemisphere of cells of the floral primordium that will give rise to the organs of the third and fourth whorls, the stamens and carpels, consistent with AG specifying a role in the specification of these two floral organs (Drews et al., 1991; Bowman et al., 1989). As WT floral development proceeds AG expression is limited to the cells of the third and fourth whorls, and by stage 7 when the anther and the filament of the stamen become distinct and the carpel primordia emerge, the AG antisense RNA probe shows a strong, uniform hybridisation signal throughout the stamen and carpel primordia (Drews et al., 1991; Bowman et al., 1991a).

In a mutant ap2 background, in situ studies show that AG expression extends to whorls one and two, causing carpelloid and staminoid characteristics respectively. In the strong ap2-2 mutant, carpelloid sepals are present in the first whorl, and AG RNA is detected from the earliest stages of whorl one development (Drews et al., 1991). In the weaker ap2-1 allele whorl one organs develop as leaf-like or slightly carpelloid organs and whorl two develop as staminoid petals, these late changes in organ fate are consistent with AG RNA accumulating later in the primordia of whorl one and two organs, as demonstrated by in situ experiments (Drews et al., 1991). These studies demonstrate that the ectopic expression of the AG gene is responsible for the ap2 phenotype, and that the AP2 gene product
negatively regulates \textit{AG} activity by repressing \textit{AG} RNA accumulation (Drews et al., 1991).

During late development of the stamens and gynoecium, when cellular differentiation takes place, the pattern of \textit{AG} expression changes from a uniform distribution throughout these primordia to become restricted to certain cell types (Bowman et al., 1991a). In the anther by stage 9, during meiosis of the sporogenous cells of the anther, \textit{AG} RNA signals are only detected at highest levels in the connective tissue of the anther, with a lower signal seen in the filament and the anther cell walls, this pattern persists through to stage 11 when pollen grains are present in the anther locules, with an additional signal seen in the nectaries. Prior to dehiscence at stage 13, \textit{AG} RNA is detected in the anther walls which at this stage are composed of only the epidermis and the endothecium (Bowman et al., 1991a). In the carpels at stage 9, the ovule primordia have developed, and \textit{AG} RNA is detected at a low level in the tissue of the ovary walls and at a higher level in each ovule primordium, this pattern persists through to stage 12 and additionally \textit{AG} RNA is detected in the stigmatic tissue, however as the ovules mature the \textit{AG} signal becomes restricted solely to the endothelium, the inner layer of the ovary integuments and the stigma (Bowman et al., 1991a). In an \textit{ap2} background the late pattern of \textit{AG} expression remains unaffected in whorl one carpels or whorl two stamens, and as in WT development \textit{AG} is restricted to specific cell types of these carpels and stamens (Bowman et al., 1991a). This data shows that \textit{AG} RNA is restricted to specific differentiated cell types, and is not detected in either the mega- or microspores, and just as early \textit{AG} expression specifies cell fate, late \textit{AG} expression may specify certain cell differentiation (Bowman et al., 1991a).

The ectopic expression of a 35S-\textit{AG} sense transgene in \textit{N. tabacum} (Mandel et al., 1992a; Kempin et al., 1993) and \textit{A. thaliana} (Mitzukami and Ma, 1992) causes a similar phenotype to \textit{ap2} mutants, specifying carpelloid petals and stamenoid petals in the first and second whorls and demonstrates the role of \textit{AG} in the specification of stamens and carpels. Third and fourth whorl organs, in these transgenic lines, developed as stamens and carpels respectively, however abnormalities were seen in later development, producing stamens that contained only a few or completely lacked pollen, or failed to dehisce, and carpels that often remained unfused with elongated stigmatic papillae and elongated or abnormal ovules (Mitzukami and Ma, 1992; Mandel et al., 1992a; Kempin et al., 1993).
Severe transformants expressing the $AG$ transgene were both female and male sterile, probably due to the ectopic expression of $AG$ in cell types that at a later stage in development do not normally express the $AG$ gene (Mitzukami and Ma, 1992; Mandel et al., 1992a). Antisense expression of the $AG$ gene in transgenic *N. tabacum*, under the control of the 35S viral promoter, showed the conversion of stamens in the third whorl to petals to varying degrees, whereas fourth whorl organs developed almost normally as carpels, although they were often not fused properly (Kempin et al., 1993). The failure of antisense $AG$ expression to cause the conversion of the fourth whorl carpel to a new floral primordium is probably a reflection of the low levels of antisense expression, being insufficient to inactivate the endogenous $AG$ levels.

However later $AG$ antisense experiments carried out in *A. thaliana* were able to demonstrate floral indeterminacy (Mitzukami and Ma, 1995), and additionally showed that two of the functions of the $AG$ gene, specification of floral organs and determinacy, were separable. Transgenic *A. thaliana* lines expressing the antisense $AG$ gene under the control of the 35S viral promoter, produced three different types of flowers producing abnormalities in the inner two whorls. Type I flowers were an $ag$-1 phenocopy showing petals in the third whorl and an indeterminate flower in the fourth whorl, type II flowers had partial conversion to sepals and petals in the third and fourth whorls respectively, and meristem indeterminacy of which the internal flowers were mainly staminoid petals and carpelloid petals, and type III flowers that showed no floral homeotic conversion but were indeterminate producing secondary flowers internal to an enlarged fourth whorl gynoecium, additional floral meristems were also visualised in the secondary gynoecium of these flowers (Mitzukami and Ma, 1995).

Examination of the $AG$ RNA levels, in 35S-$AG$ antisense lines, showed a direct correlation between the levels of $AG$ expression and the severity of the floral phenotype, with lowest levels detected in type I transgenic lines and highest levels detected in type III, which in turn were lower than WT $AG$ RNA levels (Mitzukami and Ma, 1995). These results suggest the two different functions of $AG$ activity, determinacy and floral organ specification, require different levels of $AG$ expression and that the specification of determinacy may require higher levels of $AG$ RNA (Mitzukami and Ma, 1995).

Separation of these two $AG$ functions have also been demonstrated genetically, whereby in $ag$-4 mutants carpel specification and floral determinacy
are disrupted, while in heterozygous mutants, AGMet205/ag-4, only determinacy is disrupted (Sieburth et al., 1995). One explanation proposed by Sieburth and coworkers, which agrees with the observations made by Mitzukami and Ma, implies the mutations in ag-4 and AGMet205 result in a decrease in the stability of the AG protein, with determinacy requiring highest levels, then carpel specification followed by stamen specification. The second model, proposes that both ag-4 and AGMet205 disrupt the hydrophobic face of a proposed coiled-coil motif called the K box, with the mutation in ag-4 affecting the interaction of two different cofactors required for carpel specification and determinacy, while the single amino acid change demonstrated in AGMet205 prevents the interaction of only one of these cofactors required for determinacy (Sieburth et al., 1995). Support for this second model is seen in the yeast MCM1, MADS box gene (reviewed by Herskowitz, 1989), which is also regulated by the interaction of different cofactors.

Some of the mechanisms involved in the correct temporal and spatial patterns of AG gene expression, have been proposed from the study of floral homeotic mutants. Two floral meristem identity genes (1.1.2), AP1 and LFY (Irish and Sussex, 1990; Mandel and Yanofsky, 1995; Weigel and Meyerowitz, 1993; Weigel and Nilsson 1995), may act to initiate AG expression either directly or indirectly, and two putative intermediate regulatory genes, AGL2 (Ma et al., 1991) and AGL4 (Savidge et al., 1995), have been identified. The geographical limits of AG expression in the third and fourth whorls of the developing floral meristem is controlled by the actions of two cadastral genes (1.1.3), AP2 (Bowman et al., 1991a; Drews et al., 1991) and LUG (Lui and Meyerowitz, 1995), and late in ovule development expression of the BELL gene acts to prevent AG RNA accumulation, preventing the ovules adopting a carpelloid fate (Ray et al., 1994; Modrusan et al., 1994). Additionally a downstream target of AG, has been identified, AGL5, which is activated by the ectopic expression of AG (Savidge et al., 1995). The AGL5 promoter contains a perfect match for the AG consensus binding site, to which the AG product can bind, however AGL5 expression is limited to the fourth whorl carpels and is not expressed in the stamens (Savidge et al., 1995).

The study of A. thaliana AG mutants and in situ hybridisation experiments, has demonstrated how the correct temporal and spatial regulation of the AG gene, directs many aspects of flower development, including the specification of the stamens and carpels, the control of floral determinacy, and the negative regulation of the AP2 gene. Additionally AG mRNA has been detected in specific cell types of
the stamens and gynoecium, during the later stages of development, and may control some aspects of these cells differentiation. In this work, the $AG$ coding region has been selectively expressed in a specific cell type of the anther late in development, to assess if the $AG$ gene plays a role in the late differentiation of this cell type. To investigate the role of $AG$ in late stamen differentiation, the $AG$ coding region was fused down stream of two different tapetal-specific promoters, $A6$ (Hird et al., 1993; chapter 5) and $A9$ (Paul et al., 1992; chapter 5), in both the sense and antisense orientations. These two promoters are active in the tapetum of the anther cell wall, during meiosis of the sporogenous cells and up until the formation of the microspores, when the tapetum begins to degenerate. These promoter activities almost completely coincide with detection of WT $AG$ mRNA in the anther cell walls, which is present by stage 9, during meiosis, persisting further through to when the pollen grains are present in the anther locules (Bowman et al., 1991a). These chimaeric promoter-$AG$ constructs were transformed into $A. thaliana$ and the floral morphology and anther phenotypes were evaluated in the resulting transgenic lines, to determine if the over expression or down regulation of the $AG$ gene disrupted the specification or differentiation of the tapetum at this late stage in development.

3.2 Isolation of the $AG$ gene coding region

The $AG$ gene was isolated by PCR, utilising total RNA isolated from flower buds of WT $A. thaliana$. Two floral inflorescences from Landsberg erecta were ground in 200 µl TLES buffer, and RNA was precipitated with LiCl (2.9.4). 1 µl of RNA was used to synthesise first strand cDNAs using reverse transcriptase (2.9.5). The two primers used to amplify the $AG$ coding region from the cDNA population, 5'-GACGTTAACAATGGCGTACCAATCGGAGCTAGGAG-3' and 5'-GTAAGGATCCACTCCAGGCGATTTTCCCTTCAGC-3', were designed against the $AG$ coding region (Yanofsky et al., 1990) and contain introduced Hpal and BamHI restriction enzyme sites to facilitate cloning (Fig. 3.1.b) of the PCR products. The amplified $AG$ PCR products obtained (Fig. 3.1), were cloned and subcloned into pUC19 to generate plasmids suitable for nucleotide sequencing (2.9.6), to check for the occurrence of any bp errors during PCR amplification. $AG$ PCR products were digested using Hpal and BamHI restriction enzymes and cloned into Hincll/BamHI digested pUC19 to generate pAF19, and later pAF34 (Fig. 3.2.a). Smaller subclones, were generated by digesting pAF34 and pAF19 clones with
HincII (Fig. 3.1.c) and running the products of digestion on a 0.8 % agarose gel. Each of the 5' AG HincII fragments from pAF19 and pAF34, were electroeluted and subcloned into HincII digested pUC vectors, to generate pAF32 and pAF42 respectively (Fig. 3.2.b). The HincII digested pAF19 and pAF34 vectors containing only the 3' AG coding sequence were also electroeluted and religated to generate pAF33 and pAF40 respectively (Fig. 3.2.c). Double stranded templates were synthesised for each subclone, and using both the universal primer and reverse primer of pUC19, in separate reactions, sequence analysis for each entire clone was carried out.

3.3 Generation of promoter-AG gene constructs

3.3.1 AG antisense constructs

The first AG PCR clone obtained, pAF19 (Fig. 3.2.a), was digested with BamHI and PstI and subcloned into BamHI/PstI digested pWP149 (Fig. 3.2.d) to generate a convenient A3-AG antisense pUC based plasmid pAF26 (Fig. 3.2.e). The A3 promoter was then replaced in subsequent, separate cloning experiments with the A6 and A9 promoters. pAF26 was double digested with KpnI/BamHI restriction enzymes, run on a gel, and the AG antisense pUC vector was cut from an agarose gel. The A9 and A6 promoters were then excised from pWP113 (Fig. 4.1), and pAF2 (Fig. 5.2.a) respectively, using KpnI/BamHI restriction enzymes. Digested plasmids were run on an agarose gel and A6 and A9 promoters were electroeluted and cloned separately into the AG antisense pUC vector, to generate pAF28 (Fig. 3.3.a) and pAF29 (Fig. 3.3.b), A9-AG and A9-AG antisense constructs in pUC respectively. Both promoter AG antisense constructs were then excised as Xhol fragments from pUC vectors and cloned into SalI digested pBin19, to generate pAF38 (Fig. 3.4.a), containing the A9 promoter construct, and pAF39 (Fig. 3.4.b) containing the A6 promoter construct.

Sequence analysis of the subclones of pAF19, pAF32 and pAF33, revealed the AG coding region contained two bp errors, one at position 255 and one at 725 (Fig. 3.1.d), in both cases causing a T to C base pair transition. The mispairing at position 255, caused a codon change from ATT to ACT, changing a nonpolar isoleucine side chain to an uncharged polar threonine side chain, while the C nucleotide at position 725, changed an TCA codon to CCA, changing an uncharged polar serine residue to a nonpolar proline amino acid within the AG protein. However in vitro protein synthesis of the pAF19 derived AG coding region,
from a pGEM-42 clone, utilising T7 RNA polymerase and the wheat germ translation system (Promega), generated a protein molecule approximately 30 kDa in size (Fig. 3.5), corresponding to the size of the expected AG protein. Therefore the pAF19 AG coding sequence is transcribed and translated to give a full length mRNA and protein sequence, but the mRNA contains two nucleotide changes and the protein contains two amino acid changes. Despite the two bp errors contained in the AG antisense coding region of the pAF38 and pAF39 binary vectors, these clones were still utilised for antisense analysis, as interactions between endogenous and transgenic derived mRNA species, by which antisense down regulation has been proposed, has been shown to function adequately with up to 20 % mismatch in mRNA sequence. pAF38 and pAF39 were transformed via electroporation (2.5.3) into A. tumefaciens, strain LBA4404, which was then used to transform WT C24 A. thaliana, by root mediated transformation (2.8.2). 46 individual putative AG antisense transformants were generated, 24 from the A9-AG antisense transformation, designated pAF38 A to Z (no I or J lines), and 22 from the A6-AG antisense transformation experiment, designated pAF39 A to W (no I line).

3.3.2 AG sense constructs

To determine if the over expression of AG in the tapetum affected the differentiation of this cell type, A6-AG and A9-AG transgenes were constructed. A second AG PCR product was generated, sequenced and subcloned to generate a WT AG coding sequence, to ensure the normal folding and WT function of the transgenic AG protein. The second PCR product, and pUC vector were digested with Hpa I and BamHI restriction enzymes and ligated together to generate pAF34 (Fig. 3.2.a). The 5' AG HindIII fragment from pAF34 was subcloned into HindIII digested pUC to generate pAF40 (Fig. 3.2.c), and the remaining 3' AG coding region in the HindIII digested vector was religated to generate pAF42 (Fig. 3.2.b). Sequence analysis of pAF40 and pAF42 revealed one bp change at position 32 (Fig. 3.1.d), causing a G to A bp transition. To generate one AG coding sequence that contained no bp errors, the HindIII restriction site (Fig. 3.1.e), near the 5' end of the AG gene, was used to clone the 165 bp Hind III fragment from pAF19, into the Hind III digested vector from pAF34, to generate pAF53 (Fig. 3.2.a). Sequence analysis of pAF53 confirmed this clone to contain the correct AG coding region.

To clone the AG in the sense orientation downstream of the A9 promoter, pAF53 was digested with PstI, treated with T4 polymerase (2.4.4) and then
incubated with BamHI, to release the AG coding region as a blunt end/BamHI fragment, which was cloned into pWP113 (Fig. 4.1.a), digested with NcoI, filled with T4 polymerase and digested with BamHI, to make pAF59 (Fig. 3.3.c). To construct the A6-AG sense clone, pAF53 was digested with PstI, filled with T4 polymerase and then digested with EcoRI, the AG insert was electroeluted from a agarose gel and cloned into pAF44 (an A6 expression cassette derived from pAF2, Fig. 5.2.a) digested with BamHI, T4 polymerase treated and then cut with EcoRI, to generate pAF57 (Fig. 3.3.d). Both the A6 and A9-AG sense constructs, pAF57 and pAF59, were digested from their pUC vectors with XhoI, the chimaeric gene constructs were eluted and cloned separately into SalI cut pBin19, to generate pAF68 (Fig. 3.4.c) and pAF69 (Fig. 3.4.d) respectively. pAF68 and pAF69 were transformed, via electroporation (2.5.3), into A. tumefaciens strain LBA4404, which was then used to transform WT C24 A. thaliana roots (2.8.2). 43 individual putative AG sense transformants were generated, 18 lines from the A6-AG sense transformation, designated pAF68 A to V (no I,J,N or P lines), and 25 lines from the A9-AG sense transformation experiment, designated pAF69 A to Z (no I line).

3.4 Verification of transformants and plant phenotypes

Small scale, genomic plant DNA preparations (2.9.1.1) were extracted from putative AG sense and antisense T2 A. thaliana transformants, prior to the emergence of floral inflorescences. 2 µl of genomic DNA from each transgenic line, was used in PCR reactions (2.9.2), with the AG primers (3.2), using an annealing temperature of 60 °C and an extension time of 1 min. PCR samples were visualised by agarose gel electrophoresis, in a 0.8 % agarose gel (2.4.1), and transformants were confirmed by the presence of an 833 bp band. From the total of 46 individual, putative antisense transformants, 28 individuals were subjected to PCR analysis with the AG primers, and 27 T2 lines (12 A9-AG [pAF38] and 15 A6-AG [pAF39] antisense T2 plants), demonstrated the presence of the AG gene. In the total of 43 putative sense transformants, 8 individuals were analysed by PCR with the AG primers, and 2 T2 lines (1 A9-AG [pAF69] and 1 A6-AG [pAF68] sense T2 plants) demonstrated the presence of the AG gene.

Four plants of each individual line from both the putative and confirmed AG sense and antisense T2 transformants, along with WT C24 control plants, were compost grown under artificial lighting in the greenhouse (2.7), and periodically analysed throughout floral development. All lines, from both the sense and
antisense plants, produced phenotypically normal flowers; the stamens exhibited normal morphology and produced WT quantities of fertile pollen. All lines were allowed to self-pollinate and seed production in the transgenic lines was comparable to WT control plants.

3.5 Discussion

Previous analysis of the activity of the A6 and A9 tapetal specific promoters, using promoter-reporter chimaeric constructs in transgenic plant lines, demonstrates that A6 and A9 are initially activated around meiosis, and continue until the degeneration of the tapetum (Paul et al., 1992; Hird et al., 1993; chapter 5 - this thesis). Therefore the expression of the sense and antisense AG coding region in this work, corresponds to promoter induction in floral development around stage 9, tailing off before or around stage 12, with anthesis occurring at stage 13 (Smyth et al., 1990). In situ hybridisation studies with AG indicate that at stage 7, when the anther and filament have become distinct, a uniform signal is detected throughout the stamens, however by stage 9 and beyond, expression of the AG mRNA becomes limited to specific cell types of the stamen with the highest signal in connective tissue and a lower level detected in the filament and the anther cell walls. By stage 11 the AG signal is concentrated over the connective tissue of the anther, with a much lower signal detected in the anther walls and filament (Drews et al., 1991; Bowman et al., 1991a). Therefore the AG gene is expressed in the tapetum, during the temporal expression of the A6 and A9 promoters, but at much lower levels than is previously detected in the third and fourth whorls during earlier flower development.

The AG coding sequence fused downstream of the A6 or A9 promoters, in the antisense orientation, was found to contain 2 bp changes, mis-matched during the PCR amplification of the AG cDNA. Both changes were T to C transitions, at position 245 and 715, causing amino acid changes from isoleucine to threonine, and serine to proline respectively. In vitro protein synthesis of this mutated PCR derived AG clone generated a protein molecule corresponding in size to the expected WT AG protein. Therefore the coding sequence used for the antisense transformations is transcribed to give a full length mRNA, albeit with 2 nucleotide changes, and although not ideal was transformed into A. thaliana for antisense analysis.

The transformation of A. thaliana with a sense or antisense AG coding
region driven by one of two tapetal-specific genes was designed to reveal some aspect of the late role of the AG gene in this cell type, whether required for further cellular differentiation, and if abnormal expression was capable of disrupting the normal programme of microsporogenesis. However, no visible phenotype was detected in the 27 confirmed tapetal-specific-AG antisense A. thaliana transformants or the 2 tapetal-specific-AG sense constructs.

Previous analysis of N. tabacum and A. thaliana transformants constitutively expressing the AG coding region, in the sense and antisense orientations, under the control of the 35S cauliflower mosaic virus (Mitzukami and Ma, 1992; Mitzukami and Ma, 1995; Mandel et al., 1992a; Kempin et al., 1993), has reinforced the role that AG plays in the specification of the stamens and carpels and floral determinacy, and resulting transgenic lines have shown floral homeotic conversions to varying degrees, depending on the levels of ectopic expression. 35S-AG sense transformants have caused the formation of carpelloid sepals in the first whorl and staminoid petals in the second whorl to varying degrees (Mitzukami and Ma, 1992; Mandel et al., 1992a; Kempin et al., 1993), and in some transformants both male and female sterility have been reported (Mitzukami and Ma, 1992; Mandel et al., 1992a). 35S-AG antisense transformants, have shown the conversion of the stamens to petals and indeterminacy which has also been demonstrated in transformants to differing extents, allowing the separation of the two functions of the AG gene, the control of floral organ identity and floral determinacy.

Analysis of A6 and A9 promoter fusions to the GUS and LUC reporter genes, demonstrates that both these tapetal specific promoters drive a high level of expression of chimaeric transgenes, as seen in high GUS and LUC activities produced in A. thaliana and N. tabacum transformants (Chapter 5). Therefore the levels of AG sense and antisense expression driven by the these promoters, in the transgenic A. thaliana lines generated, should have be sufficient to alter the endogenous levels of the native AG present in the tapetal cells. The lack of any visible alterations in the transgenic lines generated may be interpreted that AG has no function in tapetal development, at this late stage of cellular differentiation, and certainly did not effect the male fertility of the transgenic lines, as was observed in some severe 35S-AG sense transformants (Mandel et al., 1992a; Mitzukami and Ma, 1992).

Another possibility is that the introduced transgenes failed to work, a
scenario that was unable to be verified, as expression levels solely in the tapetum could not be determined. The failure of AG sense transformants to produce a visible phenotype, may be explained by only 2 lines being generated, a low number to guarantee or achieve a high frequency of success. However, 27 confirmed and 18 putative unchecked antisense transformants, should by probability have been adequate to reveal any phenotypic effects, judging by transgenic numbers seen in other antisense experiments (Mitzukami and Ma, 1995; Kempin et al., 1993).

In the case of the antisense transformants, a lack of any phenotype, may have been caused by the 2 bp changes that had occurred in the AG coding region, which may have been sufficient to prevent the homologous DNA or RNA interaction between the native AG and introduced transgene, thereby inhibiting the proposed mechanisms by which antisense analysis has been hypothesised to function. However, such a scenario is unlikely as antisense experimentation has been demonstrated to work well with up to 20 % mismatch.

3.6 Conclusion

The chimaeric tapetal-specific promoters fused to the AG coding region in either the sense or antisense orientation, failed to produce any visible alterations in the resulting A. thaliana transformants, which demonstrated WT morphology. These observations may be explained by a failure of the transgenes, low number of sense transgenic lines or the presence of two bp errors in the antisense AG coding region. However, both A6 and A9 promoters drive a high level of expression of chimaeric gene fusions, and these observations may be explained by AG having no role in the cellular differentiation or functionality of the tapetal cells, and the manipulation of AG expression levels in the tapetum at this stage has no detrimental or reversible effect on anther development.
Fig. 3.1 AG coding sequence, amplified by PCR.

a. ATG start codon
b. HpaI and BamHI sites introduced into the synthetic oligonucleotide primers used in cloning PCR fragment
c. HindIII site used for subcloning
d. G or T shows sites of bp error during PCR amplification
e. HindIII site used to generate pAF53.
1 GACGTTAACA ATGGCGTACC AATCGGAGCT AGGAGGAGAT TCCTCTCCCT
51 TGAGGAAAATC TGGGAGAGGA AAGATCGAAA TCAAACGGAT CGAGAACACA
101 ACGAATCGTCAATGGCGTACC TGGGAGAGGA AAGATCGAAA TCAAACGGAT CGAGAACACA
151 **AGCTT**ACGAG CTCTCTGTTC TCTGTGATGC TGAAGTCGGCA CTCATCGGTT
201 TCTCTAGCCG TGTTGCGTCTC TATGAGTACT CTAACAAACAG TGTAACAGGT
251 ACTATGGAGA GGTACAAGAA GGCAATATCG GACAAATTCA ACACCGGATC
301 GGTGCGCAGAA ATTATGCAC AGTAATATCA ACAAGAATCA GCAAATTAGC
351 **GTCAACAAAT** TATCAGCATA CAAAAACTCCA ACACCGAATT GATGGGTGAG
401 ACGATAGGGT CAATGTCTCC CAAGAGCTTC AGGAACCTGG AGGCAGATT
451 AGAGAGAAGT ATTACCGGAA TCCGATCCAA GAAAGATGAG CTTTTATTTT
501 CTGAAATCGA CTACATGCAG AAAAGAGGAAG TTGATTTGCA TAAGGATAAC
551 CAGATCTCTTC GTGCAAAGAT AGCTGAAAAT GAGAGGAAAT AGCCAGATTA
601 AAGTCTAATGC CAGGAGGATG CATCTACGAG GCAGGCTATG CCACCACCTC
651 AAACGCAATC TCAACCGTTT GATTCAOGGA ATTATTTCCA AGTCCCGGCA
701 TTGCAACCTA ACAATCACA GATATATCC GCGGTGCGGC AAGACCAAAC
751 CGCTCTCAGG TTAGTGTAA ATGGGCTGAA GGAAATGGCC TGGAGTGAGAT
801 CTTTAC
Fig. 3.2.a. 833 bp - AG coding region in pUC - pAF19/pAF34/pAF53.
pAF19 contains two bp errors at 245 and 715, while pAF34 contains one bp error at 20.
pAF53 contains the correct AG coding sequence.

Fig. 3.2.b. 368 bp - 5' HindIII subclone of the AG coding region in pUC - pAF32/pAF42.
Sequence analysis showed pAF32 to contain a T to C base pair transition at position 245, while pAF40 showed the same transition at position 20.

Fig. 3.2.c. 465 bp - 3' region of the AG coding region in pUC - pAF33/pAF40.
pAF33 and pAF40 were subcloned from pAF19 and pAF34 respectively (HindIII fragment deleted).
Sequence analysis showed pAF33 to contain a T to C base pair transition at position 715, while pAF40 showed no bp errors had occurred during PCR amplification.

Fig. 3.2.d. A3-expression cassette - pWP149.
4 kb total plasmid size.

Fig. 3.2.e. A3-AG antisense in pUC - pAF26.
4.8 kb total plasmid size.
Fig. 3.3.a. A9-AG antisense in pUC - pAF28.  
4.8 kb total plasmid size.

Fig. 3.3.b. A6-AG antisense in pUC - pAF29.  
4.8 kb total plasmid size.

Fig. 3.3.c. A9-AG sense construct in pUC - pAF59.  
4.8 kb total plasmid size.

Fig. 3.3.d. A6-AG sense construct in pUC - pAF57.  
4.8 kb total plasmid size.
Fig. 3.4.a. A9-AG antisense in pBin19 - pAF38.
12.5 kb total plasmid size.

Fig. 3.4.b. A6-AG antisense in pBin19 - pAF39.
12.5 kb total plasmid size.

Fig. 3.4.c. A6-AG sense construct in pBin19 - pAF68.
12.5 kb total plasmid size.

Fig. 3.4.d. A9-AG sense construct in pBin19 - pAF69.
12.5 kb total plasmid size.
Chapter 4
Characterisation of the A9 promoter by deletion analysis
4.1 Introduction

The A9 tapetal-specific gene was originally isolated as a cDNA clone from a B. napus anther-specific library (Scott et al., 1991a), which was then used as a probe to screen an A. thaliana genomic library, from which homologous A9 clone was isolated (Paul et al., 1992). The A. thaliana A9 gene was cloned and sequenced, and promoter sequences of 1437, 934 and 329 bp were fused upstream of the GUS reporter gene. These three A9 transgenes gave equivalent levels of GUS activity in the anthers of transgenic N. tabacum (Paul et al., 1992). In order to further characterise the A9 promoter, 5' deletion fragments of less than 329 bp were cloned upstream of the LUC reporter gene and compared to the 930 bp A9 promoter-LUC gene activity in transgenic plant lines. The 930 bp A9 promoter was cloned upstream of the LUC reporter gene, using an Ncol site containing the ATG start codon, used by both the A9 promoter and the LUC gene, creating a translational fusion. Using PCR and a convenient EcoRI site, five 5' deletion fragments of the A9 promoter were generated. These promoter fragments were also cloned upstream of the LUC reporter gene, and all constructs were transformed into WT N. tabacum, SR1 and C24 A. thaliana. Transgenic plants were assayed for normal LUC activity, to determine A9 expression patterns, and comparisons were made between each of the deletion constructs and the 930 bp promoter.

4.2 Generation of promoter-reporter gene constructs

4.2.1 Translational fusion of the full length promoter to the LUC reporter gene.

The LUC reporter gene, was cloned from pRTL-2-LUC as a Ncol/BamHI fragment into Ncol/BamHI digested pWP113 vector (Fig. 4.1.a). The resulting pJIT30 based plasmid, pAF0 (Fig. 4.1.c) was transformed by electroporation into E. coli, JM83. The full length A9 promoter-LUC fragment was cloned from pAF0, as a Xhol/Hind III fragment and ligated into Sall/HindIII digested pBin19, creating pAF1 (Fig. 4.3.a). pAF1 was transformed into E. coli, JM83 and A. tumefaciens, LBA4404 (2.5.3). Using A. tumefaciens mediated transformation pAF1 was transformed into WT, SR1 N. tabacum (2.8.1) and C24 A. thaliana (2.8.2).
4.2.2 PCR generated A9 promoter fragments, and translational fusion to the LUC gene

Using the A9 promoter sequence (Fig. 4.4), 4 forward oligonucleotide primers (A91 - A94) (Fig. 4.5) were designed to generate promoter fragments of less than 329 bp utilising PCR. At the start of each oligonucleotide sequence, the Sac I restriction enzyme site was added to facilitate cloning of the promoter fragments (Fig. 4.5). The template used for the PCR reactions to generate the A9 deletion fragments was pWP113 (Fig.4.1), a pJIT30 derived plasmid containing the full length A9 promoter (930 bp) and Camv poly A (730 bp). The reverse primer used for each PCR reaction was RSPL22 (Fig. 4.5), an oligonucleotide designed against pJIT30 sequence. PCR reactions (2.9.2) containing appropriate primers, plasmid template, polymerase and buffers, were carried out for 25 PCR cycles, with 1 cycle consisting of denaturing at 94 °C, for 1 min; annealing at 50 °C, for 1 min and an extension time of 2.5 mins at 72 °C.

The PCR reactions were visualised on a 0.8 % agarose gel, and the PCR products, approximately 1 Kb in length, were cut from the gel, electroeluted and ligated into pGEMT (Promega). The pGEMT plasmids containing the PCR products were designated pAF79 - A91/RSPL22, pAF65 - A92/RSPL22, pAF66 - A93/RSPL22 and pAF67 - A94/RSPL22 (Fig. 4.1.b). All plasmids were transformed into E.coli, strain JM83. Using the universal (UP) and reverse primer (RP) sites of pGEMT, the cloned A9 promoter fragments were sequenced, to ensure that no bp errors had occurred during PCR amplification. Once verified, promoter fragments were cloned upstream of the LUC reporter gene, in pUC. Plasmid DNA preparations of pAF79, pAF65, pAF66, pAF67 were digested with SacI/NcoI restriction enzymes to release the promoter fragments. The size of the promoter fragments generated were 130, 178, 232 and 312 bp, corresponding to A91 to A94 primers respectively. Samples were run on a 2 % agarose (Kodak) gel (Fig. 4.6). A9 promoter fragments were electroeluted and cloned into Sacl/NcoI digested pAFO, as the vector (the full length A9 promoter, now replaced by the deletion A9 fragments). The resulting clones, pAF82, pAF71, pAF72 and pAF73 (A91 - A94 respectively) (Fig. 4.1.d), were transformed into JM83. A9 promoter -LUC chimaeric fusions were cloned from their respective pUC vectors as Sacl/XhoI fragments into Sacl/Sall digested pBin19.

The resulting binary plasmids pAF83 (130 bp A9 promoter), pAF75 (178 bp A9 promoter), pAF76 (232 bp A9 promoter) and pAF77 (312 bp A9 promoter)(Fig.
4.3.b), were transformed by electroporation into *E. coli*, JM83 and *A. tumefaciens*, LBA4404. *A. tumefaciens* clones were then used to transform WT SR1, *N. tabacum* and C24, *A. thaliana* (2.8). Despite two individual *N. tabacum* transformations and a single *A. thaliana* transformation experiment, no transformants were generated for pAF75. Prior to all three transformation procedures, the transformed pAF75 LBA4404 *A. tumefaciens* culture was streaked on to agar plates, with kanamycin and streptomycin antibiotic selection (2.2.3), and a single colony was grown in liquid media. Plasmid DNA was extracted and pAF75 was digested with ScaI and HindIII, which showed an expected 2.5 kb band (gel not shown) verifying the presence of the A9 promoter (174 bp), *LUC* gene (1.65 kb) and Camv poly A tail (0.73 kb).

4.2.3 EcoRI generated A9 promoter-*LUC* gene fusion

pWP113 (Fig. 4.1.a) was digested with SmaI/EcoRV restriction enzymes, and visualised on a 0.8 % agarose gel. The vector was electroeluted and religated to form pAF81 (Fig. 4.2.a). pAF81 was digested with EcoRI/KpnI, incubated with T4 polymerase and dNTPs, to create blunt ends and religated to form pAF84 (Fig. 4.2.b). The *LUC* gene and Camv poly A were cloned from pAF0 as a Ncol/XhoI fragment and ligated into similarly digested pAF84, to create pAF85 (Fig. 4.2.c). The EcoRI generated A9 promoter fragment (65 bp in length)-*LUC*-Camv polyA fusion was cloned from pAF85 as a ScaI, XhoI fragment and ligated into ScaI, Sall digested pBin19, to create pAF86 (Fig. 4.3.c). pAF86 was transformed by electroporation into *E. coli*, JM83 and *A. tumefaciens*, LBA4404. The pAF85 *A. tumefaciens* clone was used to transformed WT,SR1 *N. tabacum* plants and C24 *A. thaliana* (2.8).

4.3 Analysis of *N. tabacum* and *A. thaliana* transformants

Small scale DNA preparations (2.9.1.1) prepared from leaf material of each putative transformant were analysed by PCR (2.9.2), using primers designed against the *npt II* gene, to verify the presence of each respective T-DNA insert. Transgenic pAF1 (chapter 5), pAF83, pAF75, pAF76, and pAF86 plant lines were assayed for LUC activity, in gametophytic and sporophytic tissue, to determine the spatial and temporal activity of each A9 promoter deletion fragment. Floral buds were harvested and sized from each transgenic line (2.10), and anthers from *N. tabacum*, or whole buds from *A. thaliana* as well as pollen samples were assayed
for LUC activity (2.10.3).

Collected data was standardised by calculating the protein concentration (2.10.4) for each sample and expressing the results (LUC activity), as units per μg protein (readings taken by the luminometer are read over a 10 s period, and therefore the results display units/μg per 10 s reading). LUC activity in buds and pollen from pAF1, pAF77, pAF76, pAF83 and pAF86 are shown in the appendix. Sporophytic tissue, from the sepal, petal, carpel, leaf, stem and root were similarly assayed, from WT SR1 and N. tabacum transformants only. LUC activity in sporophytic, transgenic tissue samples showed negative or background levels, which were comparable to that detected for WT N. tabacum plants (see appendix).
4.4 Evaluation of the A9 promoter-LUC deletion constructs

4.4.1 Comparison of LUC activity, in *N. tabacum* anther extracts, between A9 promoter deletion constructs

LUC activity in the anthers, was assessed throughout floral bud development, for the transgenic plant lines containing the full length A9-LUC transgene, pAF1, the four A9 promoter deletion fragments fused to the LUC gene, pAF77, pAF76, pAF83 and pAF86 as well as WT, SR1 *N. tabacum* (appendix). To evaluate the A9 promoter expression levels between transformants for each construct and WT, average LUC activities were compared in anthers, taken from 8 to 10 mm buds, during the time period when the A9 promoter is most active (chapter 5). The values used to calculate average LUC activity are indicated in bold in the appendix, which are depicted graphically in Fig. 4.8 and summarised in the table below, along with the calculated standard error and percentage activity with respect to the average value detected in pAF1 transformants.

<table>
<thead>
<tr>
<th>Luciferase activity (units/µg protein)</th>
<th>average</th>
<th>standard error</th>
<th>% pAF1 activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAF1</td>
<td>8049168</td>
<td>1456443</td>
<td>100</td>
</tr>
<tr>
<td>pAF77</td>
<td>4176752</td>
<td>1355502</td>
<td>52</td>
</tr>
<tr>
<td>pAF76</td>
<td>9932820</td>
<td>4272670</td>
<td>123</td>
</tr>
<tr>
<td>pAF83</td>
<td>204</td>
<td>52</td>
<td>0.003</td>
</tr>
<tr>
<td>pAF86</td>
<td>211</td>
<td>123</td>
<td>0.003</td>
</tr>
<tr>
<td>SR1 WT</td>
<td>24</td>
<td>15</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

LUC activity detected in non-transformed, WT SR1 *N. tabacum* anthers were negligible as expected, constituting background levels of luminescence produced by the anther extracts. pAF1 transformants containing the full length, 930 bp, A9 promoter gave an average value of approximately 8 million units/µg, designated as 100 % LUC activity, to enable a direct comparisons to the 5' A9 deletion fragments. In pAF77 transformants, were the promoter fragment length is 312 bp, an average value of approximately 4 million units/µg was detected, constituting 52 % of the LUC activity detected in pAF1 transformants. The average LUC activity detected in
pAF76 transformants, containing a 232 bp A9 promoter fragment, increased to almost 10 million units/µg, producing nearly a quarter more activity than that detected in pAF1 transformants. By an A9 promoter fragment length of 130 bp, for pAF83 transformants, LUC activity dropped to around 200 units/µg, showing only 0.003 % the activity detected for pAF1 transformants, and only 10 times the value seen in WT non-transformed plants. A virtually identical result was evaluated for pAF86 transformants, where the A9 promoter only contains 65 bp upstream of the ATG transcriptional start site, which also showed an average luciferase activity of around 200 units/µg protein.

4.4.2 Comparison of LUC activity, in *N. tabacum* pollen extracts, between A9 promoter deletion constructs

Unexpectedly, LUC activity was detected in pollen extracts, from A9-LUC *N. tabacum* transformants. Pollen from dehisced anthers of transgenic lines and WT SR1 *N. tabacum*, was washed four times in distilled water prior to assaying to ensure the activity detected was solely produced by the pollen grains. Average LUC activities were calculated for each A9-LUC construct (from the numbers in bold in the appendix) and are shown graphically in Fig. 4.8 and summarised in the table below along with the calculated standard error and percentage activity with respect to the average value detected in pAF1 transformants.

<table>
<thead>
<tr>
<th>Luciferase activity (units/µg protein)</th>
<th>% pAF1 activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAF1</td>
<td>191148</td>
</tr>
<tr>
<td>pAF77</td>
<td>224066</td>
</tr>
<tr>
<td>pAF76</td>
<td>535333</td>
</tr>
<tr>
<td>pAF83</td>
<td>669738</td>
</tr>
<tr>
<td>pAF86</td>
<td>371030</td>
</tr>
<tr>
<td>SR1 WT</td>
<td>4</td>
</tr>
</tbody>
</table>

Negligible, background levels, of LUC activity were detected in pollen extracts from WT SR1 *N. tabacum*. In the pollen of pAF1 transformants (930 bp A9 promoter fragment), an average LUC activity of approximately 0.2 million units/µg of protein...
was calculated, and although this represents only 2.4% of the activity seen in pAF1 bud extracts, this value is significant when compared to WT pollen extracts.
Through sequential 5' deletions of the A9 promoter, from pAF77 (312 bp) to pAF76 (312 bp) and pAF83 (232 bp), LUC activity in the pollen extracts shows a continual increase from 117 to 280 to 350% of the activity detected in pAF1 pollen extracts. LUC activity in the pollen extracts of pAF86 transformants (65 bp A9 promoter fragment), shows a decrease in activity, but is still almost twice the value of pAF1 transformants.

4.4.3 Evaluation of A9 promoter-LUC expression in A. thaliana buds and pollen

A. thaliana transformants containing the A9-LUC deletion series constructs, were briefly examined for the levels of promoter expression. A single bud, of 0.1 mm in length, as well as dehisced pollen were harvested from one of each of the transgenic line and assayed for LUC activity, the results are tabulated below.

<table>
<thead>
<tr>
<th>Luciferase activity (units/μg protein)</th>
<th>buds</th>
<th>pollen</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAF1</td>
<td>9200272</td>
<td>0</td>
</tr>
<tr>
<td>pAF77</td>
<td>3193027</td>
<td>0</td>
</tr>
<tr>
<td>pAF76</td>
<td>1113929</td>
<td>0</td>
</tr>
<tr>
<td>pAF83</td>
<td>59</td>
<td>246</td>
</tr>
<tr>
<td>pAF86</td>
<td>131</td>
<td>79</td>
</tr>
<tr>
<td>C24 WT</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The general pattern and levels of A9 expression, detected in these limited LUC assays, reflects that visualised in the N. tabacum transformants for both the bud and pollen extracts. The WT, C24 A. thaliana, showed no luciferase activity in either the bud or pollen extracts assayed. In transgenic buds, the pAF1 A. thaliana line assayed gave a LUC activity of over 9 million units/μg protein, pAF77 gave a LUC activity of over 3 million units/μg protein, while the pAF76 transformed individual gave a LUC activity of over 1 million units/μg protein. Similarly to N. tabacum transformants, the pAF83 and pAF86 A. thaliana transformants showed much
reduced levels of LUC activity in the buds where only 59 and 131 units/µg protein respectively were detected. As well as detecting similar patterns of activity in the buds between *N. tabacum* and *A. thaliana* transformants, lower levels of LUC activity were detected in pollen extracts from the pAF83 and pAF83 transformants.

4.5 Evaluation of A9 expression in transgenic *B. napus*

To establish if the A9 promoter is expressed in mature pollen of *B. napus*, Tapas ecotype, RT PCR using an A9 primer was carried out on a transgenic line expressing the A9-GUS transgene. RNA preparations were prepared from bud and dehisced pollen (2.9.4), and first strand cDNAs were synthesised (2.9.5), using 2 µl of each RNA preparation; 4 µl of 5 x RT buffer; 1 µl of 25 mM dNTPs; 8.6 µl of DEPC treated H₂O; 2 µl of 0.1 M DTT; 0.4 µl of RNAsin; 1 µl of the RACE1 primer (1 mg/ml) and 1 µl of RT (superscript). Control samples of *B. napus* bud and pollen RNA, containing 1 µl of DEPC treated H₂O instead of 1 µl RT, were also prepared. Samples were incubated at 37 °C for 1 hr, and then 1 µl of each reaction was used in separate PCR reactions (2.9.2) with the RACE2 and A9 primers at a concentration of 80 ng/µl. Samples were visualised on a 2 % agarose gel (Kodak), and the expected band of 240 bp was visualised in the PCR sample containing first strand cDNAs from *B. napus* buds with the RT present, but was not detected in either control samples or the pollen derived cDNA samples which did contain RT (Fig. 4.7). Therefore, the A9 promoter is expressed in the buds but not in the pollen of *B. napus*.

4.6 Discussion

An A9 promoter 5' deletion series, fused upstream of the *LUC* reporter gene, was constructed and transformed into *N. tabacum* and *A. thaliana*. LUC activity was detected in the floral buds of all transformants, above the background levels detected in corresponding WT plants. LUC activity was also detected in the pollen of all *N. tabacum* transformants and two of the five *A. thaliana* transformants. Subsequently, investigation of A9 promoter activity in *N. tabacum* pollen, was confirmed using X-GLUC staining of pollen from an A9-GUS transgenic line (D. Hird, Ph. D. thesis). Analysis of A9 promoter activity in *B. napus*, Tapas ecotype, from where the A9 promoter sequence was originally isolated (Paul et al., 1992), demonstrates that the A9 promoter is not active in the pollen of *B. napus* (4.5), but is activated in the pollen of all the *N. tabacum* and two of the *A. thaliana*
The 5' deletion analysis of the A9 promoter utilising fusions to the LUC gene demonstrates a similar pattern of activity in both *N. tabacum* and *A. thaliana* transformants. Floral buds from both *N. tabacum* and *A. thaliana* A9-LUC transformants, with a 3' promoter fragment of 232 bp and above consistently gave a value for LUC activity averaging in the millions of units/µg protein. In the floral buds of transformants containing a 3' promoter fragment of 130 bp or less, the LUC activity decreased to a value of one to two hundred units/µg protein. Therefore, this data indicates that the essential DNA motifs or ribosome polymerase binding elements required for the functionality of the A9 promoter lie between 130 and 232 bp upstream of the ATG start site.

Analysis of sporophytic tissue of each of the *N. tabacum* transformants, shows that the A9 promoter retains its expression within the anthers and pollen, and no activity is detected in the leaf, root, stem or other floral organs. The levels of A9 promoter activity detected in *N. tabacum* pollen grains showed a much lower, but significant value of approximately 0.2 million units/µg protein in pAF1 transformants (930 bp A9 promoter). This value increased as the promoter fragment shortened through successive 5' deletions, to give a value of around 0.7 million units/µg protein in pAF83 (130 bp A9 promoter). The promoter activity dropped to approximately 0.4 million units/µg in pAF86 (65 bp A9 promoter), still twice the value detected in pAF1 transformants. Additionally, activity in the pollen of *A. thaliana* transformants was also detected in pAF83 and at lower levels in pAF86. Therefore it appears that the successive 5' deletions of the A9 promoter enhances pollen activity up until a promoter length of 130 bp.

With the realisation that the important promoter transcriptional elements of the A9 sequence lie 130 to 232 bp upstream of the ATG start site, it has been unfortunate that the pAF75 construct, containing a 178 bp A9 promoter fragment produced neither *N. tabacum* nor *A. thaliana* transformants. Prior to a second transformation experiment in *N. tabacum*, pAF75 was checked by restriction enzyme digestion to verify the presence of the correct size insert, and the plasmid was retransformed into *A. tumefaciens* (grown with appropriate kanamycin and streptomycin selection). However no transformants were generated. One unexplored explanation may be a defect in the kanamycin gene causing a failure in the selection of transgenic individuals during plant transformation. The simplest test would be to reclone the pAF71 Sacl/ Xhol insert, containing the complete promoter-
reporter gene construct in to a new pBin19 plasmid preparation, similarly digested with Sacl/ Xhol. Retransformation into A. tumefaciens and then N. tabacum, with the formation of green putative transgenic shoots, would then indicate a fault in the kanamycin gene. If successful, evaluation of the resulting transformants would evaluate the expression levels of the 178 bp A9 promoter fragment and perhaps narrow down regions of sequence required for functional promoter activity.

4.7 Conclusions
5' deletion analysis of the A9 promoter demonstrates the essential regions of DNA sequence required for transcriptional activity to lie between 130 and 232 bp upstream of the ATG start site. The A9 promoter sequence, originally isolated from B. napus, shows A9 activity to be restricted to the tapetum in this plant species. Additionally the full length A9 promoter shows solely tapetal specific activity in A. thaliana. However, in all A9-LUC N. tabacum transformants and two of the A9-LUC A. thaliana deletion transformants, A9 promoter activity is additionally detected in mature pollen, albeit at a lower activity then that detected in the floral buds. Furthermore, pollen activity in N. tabacum and A. thaliana transformants, shows a small but notable increase through successive 5' deletion, until a promoter length of 65 bp, where A9 activity is reduced but still detectable.
Fig. 4.1.a. A9-expression cassette - pWP113. 
pJIT30 derived plasmid, containing the full length A9 
promoter (930 bp) and camv polyA tail (730 bp) 
DNA template, for PCR generation of A9 deletion fragments.

Fig. 4.1.b A9-promoter/camv polyA PCR deletion products 
cloned into the pGEMT vector - 
pAF79/pAF65/pAF66/pAF67. 
3.2 kb total plasmid size.

Fig. 4.1.c. A9 (930 bp)-LUC in pJIT30 - pAF0. 
5.7 kb total plasmid size.

Fig. 4.1.d. A9 promoter deletion fragments cloned 
upstream of the LUC gene, in pJIT30 - 
pAF82/pAF71/pAF72/pAF73. 
pAF82 - A91/RSPL22, 130 bp A9 PCR fragment 
pAF71 - A92/RSPL22, 178 bp A9 PCR fragment 
pAF72 - A93/RSPL22, 232 bp A9 PCR fragment 
pAF73 - A94/RSPL22, 312 bp A9 PCR fragment. 
Approximately 5 kb total plasmid size.
RSPL22

Amp

Approx. 2.2 Kb pJIT30 vector (b. cloned into pGEMT vector)

ori
**Fig. 4.2.a.** 930 bp A9 promoter, in pJIT30 - pAF81.
3 kb total plasmid size.

**Fig. 4.2.b.** 65 bp A9 promoter, in pJIT30 - pAF84.
pAF84 was subcloned by restriction enzyme digestion of pAF81 using KpnI and EcoRI.
Approximately 2.3 kb total plasmid size.

**Fig. 4.2.c.** 65 bp A9 promoter- *LUC* in pJIT30 - pAF85.
4.5 kb total plasmid size.
**Fig. 4.3.a.** A9 (930 bp)-LUC in pBin19 - pAF1. 13.5 kb total plasmid size.

**Fig. 4.3.b.** A9 promoter deletion fragments cloned upstream of the LUC gene, in pBin19 - pAF83/pAF75/pAF76/pAF77.
- pAF83 - A91/RSPL22, 130 bp A9 PCR fragment
- pAF75 - A92/RSPL22, 178 bp A9 PCR fragment
- pAF76 - A93/RSPL22, 232 bp A9 PCR fragment
- pAF77 - A94/RSPL22, 312 bp A9 PCR fragment
Approximately 12.8 kb total plasmid size.

**Fig. 4.3.c.** 65 bp A9 promoter-LUC in pBin19 - pAF86. 12.6 kb total plasmid size.
**Fig. 4.4** DNA sequence of the A9 promoter from *A. thaliana*.

**a.** Underlined nucleotides, indicate the sequence used to synthesise the four 5’ oligonucleotide primers used to generate the A9 promoter PCR deletion fragments, giving rise to 312, 232, 178 and 130 bp promoter fragments.

**b.** EcoRI site used to generate the 65 bp A9 promoter fragment, indicated in bold.
Fig. 4.5 A9 promoter oligonucleotide primers.

Sequence of oligonucleotide primers used to generate the A9 promoter, deletion fragments by PCR.

Underlined nucleotides indicates the introduced Sacl restriction enzyme site, used to facilitate cloning of the promoter fragments generated.
A91  5' ATA GAG CTC GAC CTA ATT ATT CTC GAA GCC C 3'
A92  5' ATA GAG CTC GAA GTA ACT AGA TCA AGA TCA C 3'
A93  5' ATA GAG CTC CAC ACA CTT TGT TAT TTC CCC T 3'
A94  5' ATA GAG CTC GGG TTTTGA ATA TGT TGT GGA G 3'
RSPL22 5' CAC ACA GGA AAC AGC TAT GAC C 3'
**Fig. 4.6.** Agarose gel electrophoresis showing A9-promoter deletion fragment products.

(top figure)

pGEMT vectors pAF79, pAF65, pAF66 and pAF67 were enzymatically digested with Sacl, and Ncol to release the A9 promoter deletion fragments.

- **lane 1** 1 Kb λ DNA ladder
- **lane 2** pAF67 - Sacl/Ncol, 312 bp A9 promoter
- **lane 3** pAF66 - Sacl/Ncol, 232 bp A9 promoter
- **lane 4** pAF65 - Sacl/Ncol, 178 bp A9 promoter
- **lane 5** pAF79 - Sacl/Ncol, 130 bp A9 promoter.

**Fig. 4.7** Agarose gel of RT PCR from *B. napus* indicating the presence of the A9 transcript in buds, but not pollen.

(bottom figure)

2 % agarose gel, showing the RT PCR results from bud and pollen RNA extracts from *B. napus*, ecotype Tapas,

- **lane 1** bud RNA + RT, shows 240 bp RT PCR fragment
- **lane 2** bud RNA - RT control
- **lane 3** 1 Kb λ DNA ladder
- **lane 4** pollen RNA + RT
- **lane 5** pollen RNA - RT control.
Fig. 4.8 LUC activity detected in anther and pollen extracts from transgenic *N. tabacum* lines, expressing A9 promoter, deletion fragments, fused to the *LUC* reporter gene.
A9 promoter fragment length (bp)  LUC activity (millions units/µg protein)
Chapter 5
Analysis of A6 and A9 promoter-reporter
gene expression in *N. tabacum* and *A. thaliana*
5.1 Introduction

The A6 and A9 cDNA clones, initially isolated from a B. napus anther-specific cDNA library (Scott et al., 1991b), were subsequently used to isolate homologous clones from an A. thaliana library (Paul et al., 1992; Hird et al., 1993). Promoter fragments isolated from the A. thaliana clones were fused to both the GUS reporter gene (Jefferson et al., 1987) and to the coding sequence of the cytotoxic RNase barnase gene of B. amyloliquefaciens (Hartley, 1988). Promoter-GUS and barnase fusions were transformed into N. tabacum, to reconfirm the temporal and spatial activities of the A6 and A9 promoters originally evaluated from endogenous B. napus RNA transcripts (Scott et al., 1991b; Paul et al., 1992; Hird et al., 1993).

N. tabacum plants transgenic for the chimaeric A6 or A9 promoter-GUS fusions were analysed in a series of buds of different sizes, one anther from each bud was assayed for GUS activity, while a second anther was used to determine the developmental stage by light microscopy. GUS activities showed that both A6 and A9 are initially expressed around sporogenous cell meiosis, show a dramatic increase reaching a plateau around microspore release, and then fall to cease expression prior to microspore mitosis (Paul et al., 1992; Hird et al., 1993). Transgenic N. tabacum expressing A6 or A9-barnase chimaeric constructs were male sterile due to the destruction of the tapetum, caused by the presence of cytotoxic RNase protein transcribed by promoter activity. Examination of a sequential series of buds from these transgenic lines, indicated that A9 is active in the tapetum prior to sporogenous cell meiosis and A6 is active later following the completion of meiosis but prior to microspore release (Paul et al., 1992; Hird et al., 1993).

The GUS and barnase data suggested the possibility to devise a strategy whereby the A6 and A9 promoters could be used to divide the stamen differentiation pathway into temporal windows, whereby A9 promoter activity would delimit the start of meiosis and A6 the end. These windows generated by tapetal-specific promoter gene expression could then be used to screen for mutations that lie within these specified time points. The designed strategy was to generate homozygous transgenic seed of A. thaliana, transformed with a chimaeric, double promoter-reporter transgene, whereby the A6 and A9 promoters were fused to two different reporter genes, either GUS or LUC, to differentiate between the activities of each promoter. Transgenic, homozygous
seed would then be mutagenised and the resulting M2 population would be screened for a down regulation of the A6 promoter, by assaying for the inactivity of the reporter gene fused to this promoter. Individuals showing no A6 activity, would then be screened for A9 activity, assaying for reporter gene activity fused to A9. If no A9 activity was detected then the mutation would be classified as pre-meiotic, whereas detection of A9 activity would classify the mutation as meiotic. This approach would identify mutants in the early stages of stamen differentiation, defective in specific aspects of tapetum differentiation.

In order to determine whether the expression patterns of the two tapetum-specific genes, $A_6$ and $A_9$, could be used to define temporal windows along the stamen differentiation pathway, the promoters of both these genes were fused to the coding regions of either the LUC or GUS reporter genes. Single chimaeric constructs comprising one promoter-reporter gene construct (5.3) and double constructs containing both promoters fused to different reporter genes (5.4) were transformed into $N.\ tabacum$ and $A.\ thaliana$ and appropriate assays were carried out on sized buds (2.10). Previous analysis in $B.\ napus$, has demonstrated the allometric relationship between bud and anther length and developmental stage (Scott et al., 1991b). Similarly the correlation between $N.\ tabacum$ or $A.\ thaliana$ bud length and developmental stage enable a comparison of the temporal activity of the A6 and A9 promoters (5.2).

In addition to detecting promoter activity by assaying for GUS or LUC activity, a visual screen to detect the activity of the A6 promoter utilising the $TMS2$ gene was evaluated in $A.\ thaliana$ (5.5). The $TMS2$ gene (Klee et al., 1987) encodes an amino hydrolase, $iaaH$, which converts biologically inactive auxin amides into active auxins which are toxic to plants at elevated concentrations. The $TMS2$ gene amino hydrolase product is utilised by bacteria in an auxin biosynthesis pathway, and catalyses IAM to the phytohormone IAA. Transgenic plants containing the $TMS2$ gene are phenotypically normal, as they do not contain the endogenous substrate, however exposure to IAM, leads to the production of IAA which causes an obvious change in tissue or organ development as shown in $N.\ tabacum$ seedlings (Budar et al., 1986); petunia leaf disk explants (Klee et al., 1987) and $A.\ thaliana$ seedlings (Karlin-Neumann et al., 1991).

Based on this expectation, transgenic $A6-TMS2$ plants could show a phenotypic change in the anther or flower development, when sprayed with
IAM. If successful, the mutagenesis of transgenic A6-TMS2 seeds would facilitate the positive selection of mutant lines carrying a mutation upstream of A6, determined visually by the presence of normal flowers or anthers. This strategy would eliminate the need to assay each and every M2 individual for A6-reporter gene activity, providing a visual and extremely labour saving approach.

5.2 Correlation of N. tabacum and A. thaliana bud length and developmental stage of the microsporangium

To establish the relationship between bud length and developmental stage of the anther, WT buds were harvested from N. tabacum, SR1, and A. thaliana, C24. Individual bud lengths were measured from the base of the pedicel to the tip of the sepals. The small size of A. thaliana buds, required these to be measured under magnification. The sized buds were then gently squashed, stained with aceto-orcein and observed using light microscopy to determine the developmental stage. In both N. tabacum and A. thaliana a variation in size was seen for each landmark stage, but on average meiosis occurs in 8 mm buds in N. tabacum and 0.5 mm in A. thaliana. Similarly tetrads are visualised at 9 mm and 0.6 mm and microspore release occurs at 10 mm and 0.7 mm respectively (Fig. 5.1).

5.3 Construction of single promoter-LUC fusion plasmids

5.3.1 Construction of chimaeric A9-LUC constructs

A 934 bp Ncol/BamHI fragment of the A9 promoter from pWP113, was cloned into Ncol/BamHI digested pRTL-2-LUC, a pUC based vector containing the LUC gene to form pAF0 (Fig. 4.1.c). The A9-LUC Xhol/HindIII fragment was cloned from pAF0 into Sall/HindIII digested pBin19 to generate pAF1 (Fig. 4.3.a). pAF1 was transformed into A. tumefaciens, LBA4404 (2.5.3) which was used to transform SR1 N. tabacum leaf explants (2.8.1).

5.3.2 Construction of chimaeric A6-LUC constructs

A 885 bp Xbal/KpnI A6 promoter fragment was cloned from pWP146 into Xbal/KpnI digested pJIT30 to create pAF2 (Fig. 5.2.a), an A6 expression cassette. The LUC gene was cloned from pJIT27 as a Stu1/EcoICRI fragment and cloned into Smal digested pAF2 to generate pAF3 (Fig. 5.2.b). pAF3 was digested with BamHI, electroeluted from an agarose gel and religated to form
pAF5 (Fig. 5.3.a). The XhoI A6-LUC fragment digested from pAF5 was cloned into Sall digested pBin19, forming pAF10 (Fig. 5.5.a). pAF10 was transformed into SR1 N. tabacum via A. tumefaciens mediated transformation (2.8.1).

5.3.3 Temporal expression patterns of promoter-LUC constructs in transgenic N. tabacum anthers

LUC assays (2.10.3) were carried out on anthers from sized buds of pAF1 (A9-LUC transgene, Fig. 4.3.a) and pAF10 (A6-LUC transgene, Fig. 5.5.a) N. tabacum transformants. LUC activity is first detected in the anthers of pAF1-9, at a bud length of 7 mm, while in pAF1-4 and pAF1-11 transformants, no LUC activity is detected until a bud length of 8 mm (Fig. 5.6.a). LUC activity, in pAF1 transformants gradually increases until a bud length of approximately 12 mm, after which time the activity begins to tail off. In pAF10-1 and pAF10-3 N. tabacum transformants, a sharp peak of LUC activity was seen in 10 mm buds (Fig. 5.6.b). However, ten fold lower LUC activity was detected in pAF10 transformants prior to a bud length of 10 mm and in the pAF10-4 transformant, LUC activity was detected in anthers taken from a 5 mm bud (Fig. 5.6.b).

5.4 Construction of double promoter-reporter fusion plasmids

5.4.1 Construction of chimaeric A6-LUC, A9-GUS constructs

A9-GUS was digested from pWP104 as an PstI - partial EcoRI digest, the fragment was treated with T4 polymerase, to generate blunt ends, and cloned into HincII digested pUC19 to form pAF4 (Fig. 5.2.c). A9-GUS was cloned from pAF4 as an KpnI/HindIII fragment into KpnI/HindIII digested pBin19 generating pAF6 (Fig. 5.4.a). A6-LUC was digested from pAF5 (Fig. 5.3.a) as an XhoI fragment and cloned into SalI cut pAF6 to generate pAF7 (Fig. 5.4.b), a binary vector containing A6-LUC/ A9-GUS in tandem.

5.4.2 Construction of chimaeric A6-GUS, A9-LUC constructs

pAF0 was digested with Sall and treated with T4 polymerase to generate blunt ends and eliminate the Sall site. The resulting blunt ended plasmid was religated to form pAF8 (Fig. 5.3.b). A6-GUS was digested from pBINA6-GUS (D. Hird) as a Sall fragment and cloned into XhoI cut pAF8, generating two separate clones, pAF9 (Fig. 5.4.c) and pAF11 (Fig. 5.5.b). In pAF9 clone, the A6-GUS fragment had inserted so that both promoters were facing the same way, in
tandem, while in the second clone, pAF11, the two promoters were facing in opposite directions.

5.4.2.1 Temporal expression patterns of promoter-reporter double constructs in transgenic *N. tabacum* anthers

Promoter-reporter double constructs, pAF7 (Fig. 5.4.b), pAF9 (Fig. 5.4.c) and pAF11 (Fig. 5.5.b) were transformed into *A. tumefaciens* (2.5.3) and *SR1 N. tabacum* (2.8.1). Anthers from a series of sized buds, from each transgenic line, were assayed for GUS (2.10.2) and LUC (2.10.3) activity. In pAF7 *N. tabacum* transformants, GUS and LUC activity is first detected at a low level in 8 mm buds (Fig. 5.7). In the pAF7-4 transformant, a sharp increase in LUC activity is detected in 10 mm buds, as demonstrated in pAF10 transformants. In pAF9-2 *N. tabacum*, GUS activity is first detected at a bud length of 7 mm, and LUC activity starts at 8 mm. A reciprocal pattern is seen in pAF9-4, with LUC activity being detected first at 7 mm, with GUS activity starting at 8 mm. In pAF9-11, GUS and LUC activities are detected at the same time in the anthers, at a bud length of 8 mm (Fig. 5.8).

In pAF11 *N. tabacum* transformants, the transgene present is A9-LUC/A6-GUS, and the promoter-reporter constructs face in opposite directions on the T-DNA insert (Fig. 5.5.b). Three pAF11 transformants, pAF11-5, pAF11-6 and pAF11-7, show that initial GUS and LUC activity is detected simultaneously, at a bud length of 8 mm (Fig. 5.9). A fourth transformant, pAF11-3, shows initial LUC activity at a bud length of 7 mm, prior to GUS activity at a bud length of 8 mm (Fig. 5.9).

To evaluate if the pattern of reporter gene expression in the primary transformants was inherited by progeny lines, two pAF11 T2 plants from pAF11-3 and pAF11-5 were examined for GUS and LUC activity in the anthers of sized buds. The pAF11-3 primary transformant showed LUC activity detected in 7 mm buds prior to GUS expression at 8 mm buds, in the secondary transformant this pattern of reporter gene expression changed, and both reporter genes were initially detected in 8 mm buds (Fig. 5.10). In pAF11-5 primary and secondary transformants, LUC activity is detected before GUS activity, but in the primary transformant this activity is seen in a smaller bud length than the secondary transformant (Fig. 5.10). In both pAF11-3 and pAF11-5, the parental and sibling lines show a similar level of LUC activity in the anthers, whereas the GUS
activity either shows a large fold increase (pAF11-3 T2) or decrease (pAF11-5 T2) compared to the parental line (Fig. 5.10).

5.4.2.2 Temporal expression patterns of promoter-GUS single constructs in transgenic A. thaliana buds

A. thaliana seed, transgenic for A6 or A9-GUS, were supplied by Biochem (France). Homozygous lines were identified for each construct, namely A6-17 and A9-127. Both lines were assayed for GUS activity, in sized buds collected from individual plants. GUS assays on transgenic A. thaliana, detected A6 activity in 0.45 mm long buds, prior to A9 activity first detected in 0.5 mm buds (Fig. 5.11).

5.4.2.3 Temporal expression patterns of A6-GUS, A9-LUC constructs in transgenic A. thaliana buds

Three pAF11 A. thaliana transformants, lines B, T and S, were assayed for GUS and LUC activity in a series of sized buds, harvested from individual plants. In lines B and S, A9 activity was initially detected in 0.45 mm and 0.4 mm buds respectively, prior to A6 activity detected later at 0.55 and 0.5 mm respectively (Fig. 5.12). The third transformant, line T, showed simultaneous detection of A6 and A9 activity in a bud length of 0.45 mm. Line T, showed a pronounced increase in GUS activity, driven by the A6 promoter, at a bud length of 0.6 mm (Fig. 5.12).

5.5 Construction of chimaeric A6-TMS2 gene constructs

The TMS2 gene, obtained from Monsanto, was cut from pMON544 as a BamHI fragment and cloned into BamHI cut pAF2 to generate pAF18 (Fig. 5.3.c). The A6-TMS2 fragment was cut from pAF18 as an XhoI fragment and cloned into Sall cut pBin19 which had been treated with phosphatase, to create pAF25 (Fig. 5.5.c). pAF25 was transformed into A. tumefaciens, LBA4404 (2.5.3) which was used to transform A. thaliana by root mediated transformation (2.8.2).

5.5.1 Effect of spaying IAM on A6-TMS2 transgenic A. thaliana

A. thaliana seed harvested from putative primary transformants for the A6-TMS2 transgene, were sown onto a 3:1 mixture of compost and sand, and
allowed to grow until floral inflorescences were apparent. Small scale genomic DNA extractions were performed on leaf material (2.3.1), and extracts were tested by PCR (2.9.2) for the kanamycin resistance conferring *nptII* gene, to confirm the presence of the transgene. Four individual A6-TMS2 transgenic lines were chosen for evaluation of spraying with IAM, lines A, T, Q and Y. Three transgenic plants for each line, were sprayed with different concentrations of IAM, 5 mg/L, 50 mg/L and 500 mg/L, along with three control WT *A. thaliana* plants, sown and grown in parallel. Plants were sprayed every day (approximately 200 ml/plant), until mature flowers were produced. Examination of *A. thaliana* plants, exposed to IAM, by eye revealed no visual defects occurring within the mature flowers. Anthers were dissected from open flowers and examined under a dissecting microscope. No differences in floral, or anther morphology were seen in any of the sprayed, transgenic lines, or WT controls. All IAM sprayed plants exhibited normal vegetative and floral growth, and produced siliques which contained seeds numbers comparable to WT *A. thaliana* grown alongside, under the same growth conditions.

5.6 Discussion

Previously, fluorimetric GUS assays carried out on A6-GUS and A9-GUS transgenic *N. tabacum* anthers (Paul et al., 1992; Hird et al., 1993), has shown that A9 and A6 activity is initiated during early sporogenous cell meiosis, at an anther length of 2 mm, equivalent to a bud length of 8 mm. Additionally, *N. tabacum* A9 and A6-barnase data showed complete destruction of the tapetum by an anther length of 1.8 and 3 mm respectively (Paul et al., 1992; Hird et al., 1993). The barnase data, indicated that A9 is active before sporogenous cell meiosis, and A6 is active after the completion of meiosis but immediately prior to microspore release. The promoter-barnase data can be considered more sensitive indicators of the onset of promoter activity than promoter fusions to GUS, and predicts defined temporal windows during microsporogenesis, between A6 and A9 initial gene expression.

Further investigation of the temporal expression patterns of A6 and A9, was carried out by assaying anthers, for GUS and LUC activity, from *N. tabacum* transformed with pAF1, pAF10, pAF7, pAF9 and pAF11 (5.4.2.1; 5.4.2.2 and 5.4.2.3). The GUS and LUC data gathered from *N. tabacum* and *A. thaliana* transformants, showed the activation of A6 and A9 can vary. In some
transformants the promoters appear to be switched on simultaneously, as in pAF7-4 (Fig. 5.7) or pAF11-5 (Fig. 5.9). In other transformants, pAF9-4 (Fig. 5.8) or pAF11-3 (Fig. 5.9), A9 is activated before A6, while other transgenic lines, pAF9-2 or pAF9-11 (Fig. 5.8), demonstrate the reverse.

In some transgenic lines, very low initial reporter gene activity was detected at a time when the promoters were not expected to be activated, which may be partly due to human errors or inadequacies in the reporter gene assay system. The data from pAF1 and pAF10 *N. tabacum* transformants, does not reflect previous *bamase* data, whose sensitivity is such that a few molecules of RNase are sufficiently toxic to cause cell death, highlighting the reduced sensitivity of the *LUC* and *GUS* reporter genes which are insufficient to be able to define clear and reproducible temporal windows during the short period of time spanning meiosis.

However, one distinct feature of A6 activity, in both *N. tabacum* and *A. thaliana* transformants, was the sharp increase in reporter gene expression, seen at a developmental stage prior to microspore release from the tetrads (Fig. 5.6.b; Fig. 5.7.b; Fig 5.9.b and Fig. 5.12), in agreement with previous observations and the suggestion that A6 may be involved in the dissolution of callose that surrounds the tetrads (Hird et al., 1993).

The third reporter gene, *TMS2*, fused to the A6 promoter, was unable to demonstrate the catalytic action of encoded amino hydrolase, *iaaH*, to convert exogenous applications of IAM to IAA within the tapetum. *A. thaliana* lines, transgenic for the A6-*TMS2* construct, developed normal flowers and produced fertile seeds, after spraying with IAM, indicating that the tapetum remains intact and functional in these lines, and not been disrupted by the production of IAA.

The levels of *TMS2* RNA should have been evaluated, by northern blotting, in the lines used for spraying experiments to check the presence of the *TMS2* transcript, although plants were checked by PCR analysis for the presence of the T-DNA insert. The concentration of IAM sprayed on the transgenic lines should have been adequate to produce a response, as previously it has been shown that applications of 1 μm of IAM, are sufficient to produce abnormal seedlings grown on agar medium, transgenic for the *cab140* phytochrome promoter fused to the *TMS2* gene (Karlin-Neumann et al., 1991).

It is more likely that the method and timing of exogenous application of IAM is at fault, and sufficient levels of IAM may not have reached the tapetal
cells during the window of A6 expression. Another possibility is that the IAM simply leached through the compost and was not taken up by the plants, the application of a suitable wetting agent such as 1% sapogenat (available from AgrEvo U.K.), and spraying from seedlings may circumvent these problem. If however, the IAM substrate did reach the tapetal cells in these transgenic lines, then an alternative explanation may be that the IAA levels produced may not have been sufficient to provoke an alteration either in these or adjacent cells. However due to the high activity of the A6 promoter, this explanation is unlikely. One solution may have been to use a more toxic auxin byproduct, such as α-NAA, catalysed by the action of the TMS2 gene on α-NAM. Whichever alternative approach was utilised, the strategy should have been first evaluated by growing transgenic lines on selective media containing the inactive auxin substrates at various concentrations, to eliminate the possibility that the plants failed to take up enough substrate due to lack of exposure. Once successful at this stage, it would then have been possible to devise a strategy, whereby the inactive auxin was applied by a spraying technique.

5.7 Conclusion

It was proposed that the two tapetal-specific promoters, A6 and A9, could be utilised, when fused to either the GUS or LUC gene, to provide temporal windows during the early stages of sporogenesis, which could be identified and delimited by assaying for the activity or inactivity of the GUS and LUC genes. This strategy proved unsatisfactory as no clear or reproducible windows could be ascertained, despite previous data obtained from N. tabacum promoter-barnase transformants suggesting the contrary.

The strategy to devise a visual screen, using the TMS2 gene, to positively select for mutations upstream of A6 activity was also unsatisfactory. Although the potential for such a screening system is there, and further minimal experimentation with other substrates in culture conditions may prove successful.
Fig. 5.1 Correlation between bud length and developmental stage of the microsporangium in *N. tabacum* and *A. thaliana*.

Abbreviations: **Me**, meiosis; **T**, tetrads; **EM**, early microspores.
**Fig. 5.2.a** A6-expression cassette - pAF2.  
4 kb total plasmid size.

**Fig. 5.2.b** A6-\textit{LUC} in pUC - pAF3.  
5.7 kb total plasmid size.

**Fig. 5.2.c** A9-\textit{GUS} in pUC - pAF4.  
6.4 kb total plasmid size.
**Fig. 5.3.a** A6-\textit{LUC} in pUC - pAF5. 5.7 kb total plasmid size.

**Fig. 5.3.b** A9-\textit{LUC} in pUC - pAF8. 5.7 kb total plasmid size.

**Fig. 5.3.c** A6-\textit{TMS2} in pUC - pAF18. 6 kb total plasmid size.
**Fig. 5.4.a** A9-\textit{GUS} in pBin19 - pAF6.
14 kb total plasmid size.

**Fig. 5.4.b** A6-\textit{LUC} /A9-\textit{GUS} in pBin19 - pAF7.
17 kb total plasmid size.

**Fig. 5.4.c** A6-\textit{GUS} /A9-\textit{LUC} in pBin19 - pAF9.
17 kb total plasmid size.
Fig. 5.5.a A6-\textit{LUC} in pBin19 - pAF10.  
13.5 kb total plasmid size.

Fig. 5.5.b A6-\textit{GUS/A9-LUC} in pBin19 - pAF11.  
17 kb total plasmid size.

Fig. 5.5.c A6-\textit{TMS2} in pBin19 - pAF25.  
13.5 kb total plasmid size.
Fig. 5.6.a LUC activity in pAF1 *N. tabacum* T1 plants.

Reporter gene activity in anthers taken from sized buds of three independent pAF1 primary tobacco transformants, pAF1-4, pAF1-9 and pAF1-11, were the LUC transgene is driven by the A9 promoter.

Fig. 5.6.b LUC activity in pAF10 *N. tabacum* T1 plants.

Reporter gene activity in anthers taken from sized buds of four independent pAF10 primary tobacco transformants, pAF10-1, pAF10-3, pAF10-4 and pAF10-5, were the transgene present is A6-*LUC*.
**Fig. 5.7** LUC and GUS activity in pAF7 *N. tabacum* T1 plants.

Reporter gene activity in anthers taken from sized buds of two independent pAF7 primary tobacco transformants, pAF7-4 and pAF7-5, where the transgene present is $A6\text{-}LUC/A9\text{-}GUS$ and both promoters are in tandem on the T-DNA insert,

a. LUC activity in anthers of pAF7 transformants  
b. GUS activity in anthers of pAF7 transformants.
Fig. 5.8 LUC and GUS activity in pAF9 *N. tabacum* T1 plants.

Reporter gene activity in anthers taken from sized buds of three independent pAF9 primary *N. tabacum* transformants, pAF9-2, pAF9-4 and pAF9-11, where the transgene present is A9-\textit{LUC}/A6-\textit{GUS} and both promoters are in tandem on the T-DNA insert,

\begin{itemize}
  \item[a.] LUC activity in anthers of pAF9 transformants
  \item[b.] GUS activity in anthers of pAF9 transformants.
\end{itemize}
Fig. 5.9 LUC and GUS activity in pAF11 *N. tabacum* T1 plants.

Reporter gene activity in anthers taken from sized buds of four independent pAF11 primary tobacco transformants, pAF11-3, pAF11-5, pAF11-6 and pAF11-7, where the transgene present is A9-*LUC*/A6-*GUS* and both promoters face in opposite directions on the T-DNA insert,

a. LUC activity in anthers of pAF11 transformants
b. GUS activity in anthers of pAF11 transformants.
A6-GUS activity (nmoles MU/min/mg protein) | A9-luciferase activity (millions units/pg protein)

bud length (mm)

- pAF11-3 LUC
- pAF11-5 LUC
- pAF11-6 LUC
- pAF11-7 LUC

bud length (mm)

- pAF11-3 GUS
- pAF11-5 GUS
- pAF11-6 GUS
- pAF11-7 GUS
Fig. 5.10 Comparison of LUC and GUS activity, between pAF11 *N. tabacum* T1 and T2 plants.

Reporter gene activity in anthers taken from sized buds of two independent pAF11 T1 *N. tabacum* plants, pAF11-3 and pAF11-5, and their T2 progeny,

a. LUC activity in pAF11-3 primary transformant, and a sibling line pAF11-3 T2
b. GUS activity in pAF11-3 primary transformant, and a sibling line pAF11-3 T2
a. LUC activity in pAF11-5 primary transformant, and a sibling line pAF11-3 T2
b. GUS activity in pAF11-5 primary transformant, and a sibling line pAF11-5 T2.
Fig. 5.11 GUS activity in the floral buds of A6-17 and A9-127 A. thaliana transformants.
Fig. 5.12 LUC and GUS activity in pAF11 A. thaliana transformants.
Chapter 6

Generation and characterisation of

*Arabidopsis thaliana* stamen development mutants
6.1 Introduction

The study of developmental mutants, which disrupt the normal course of events during growth, and the isolation and characterisation of WT gene products responsible, is one approach to elucidating gene function and the mechanisms regulating the differentiation and metabolic functions of an organism. Mutants can be generated using a variety of physical and chemical agents, and desirable individuals arising in the resulting population can be identified, via visual, chemical or genetic means. The analysis of genes utilising mutants overcomes the problems encountered in screening differential libraries, where the gene of interest is not highly expressed, and the mRNA levels present in a library are in low abundance, providing the genetic locus is susceptible to mutagenic attack. Additionally, widely available classical and molecular techniques facilitate the characterisation of the mutant phenotype, and importantly allow the direct observation of the effect of the mutagenised gene on the development of the organism. The availability of mutant lines also enables the study of double mutants or genetic chimaeras, which contribute to the dissection and understanding of complex genetic pathways.

Mutagenesis studies have been undertaken in model animal and plant species, such as *D. melanogaster* and *A. thaliana*, where the haploid genome size is small, with low levels of interspersed repetitive DNA, a prerequisite if mutagenised genes are to be isolated by map-based cloning techniques. Other criteria required of a model species for mutagenesis, include a short generation time and small physical size, to assist in generating a large M2 population, as well as the suitability for molecular and classical manipulation, to aid in the characterisation and isolation of WT genes corresponding to a mutant phenotype. Previous genetic characterisation and the availability of existing mutants provide invaluable resources for the genetic analysis of a new mutation, required for allelism test crosses and mapping experiments. Such mutant analysis, has given an insight into the processes that regulate cell-type determination, differentiation and metabolism and has led to the proposal of genetic models in many aspect of development. Extrapolation of the underlying genetic principles uncovered in such model species may be applicable to varying degrees in other higher plants and animals, where genetic analysis is difficult.

The study of floral mutants of *A. thaliana* has also led to a model which attempts to explain floral determination and differentiation. This ABC model (1.1.1) involves a cascade of gene expression and signalling systems (reviewed by Coen
and Carpenter, 1993; Okamuro et al., 1993; Ma, 1994; Okada and Shimura, 1994). Gene expression controls the initial transition of the vegetative meristem to the inflorescence meristem, from which floral meristems are initiated by the actions of floral meristem identity genes (1.1.2). The floral meristem identity genes positively regulate the expression of the floral homeotic genes (1.1.1) and the cadastral genes (1.1.3), which act antagonistically to define the boundaries of action of floral homeotic gene expression. The restriction of the floral homeotic genes to specific cell regions of the floral primordium, which can overlap, enables the different combinations of the floral homeotic genes to specify the identity of the four different types of floral organs.

In *A. thaliana*, mutants have been generated using a variety of physical and chemical mutagens, such as T-DNA insertional mutagenesis, transposon tagging, chemical and irradiation mutagenesis. Comparisons between the chemical and irradiation mutagens have demonstrated differences in efficiency; the level of mutation frequency obtained at tolerable levels of lethality or sterility. It is also known that conditions of irradiation, including dose, dose rate and oxygen and moisture content of the target tissue, can effect the outcome of mutagenesis in plants (Underbrink et al., 1970).

The study of *erectoides* and *eceriferum* mutants, induced in barley using ionising radiations and chemical mutagens (Lundqvist and Von Wettstein, 1962), showed the sparsely ionising radiation produced by gamma and X-rays, had nearly three times the mutation rate with *erectoides* than *eceriferum* mutants. In contrast densely ionising radiation produced by fast-neutrons had up to a twenty fold higher induction of *eceriferum* mutants. Similarly, the mutation frequencies of *eceriferum* mutants in *A. thaliana* (Koornneef et al., 1982), were found to be of a comparable order of magnitude. In both the barley and *A. thaliana* studies (Lundqvist and Von Wettstein, 1962; Koornneef et al., 1982), the use of ethylmethane sulphonate (EMS) as a mutagen, rather than ionising radiation, was found to generate higher mutation frequencies, with a three fold increase seen in Barley *eceriferum* mutants, and a four fold increase in *A. thaliana* embryonic chlorophyll mutants. Chemical and irradiation mutagens were also found to generate different mutant spectra, demonstrated by certain loci to be preferentially and perhaps exclusively induced by certain types of mutagens, with the *cer-i* locus being solely isolated using ionising radiation and the *cer-j* locus with chemical mutagens (Lundqvist and Von Wettstein, 1962). Other specific loci, found in gibberellin-sensitive or hypocotyl
mutants of *A. thaliana*, showed highest average mutation frequencies, indicating that some loci are more readily mutated than others (Koornneef et al., 1982).

Each mutagenic agent, by virtue of its mode of action, causes a different type of DNA lesion, including point mutations, insertions, inversions, translocations and deletions. Subsequently, the ease and technique employed to isolate the mutagenised gene in question, can depend on the type of lesion present, and whether the gene is tagged or not. Insertional mutagenesis or gene tagging, is based on the integration of a foreign sequence of DNA into a gene, both mutating the gene and serving as a marker enabling the isolation of the flanking gene sequence. Strategies for insertional mutagenesis, have employed the activities of mobile transposable elements, and the integration of T-DNA inserts, achieved during plant transformation using *A. tumefaciens*.

The maize Enhancer-Inhibitor transposable element system, has been used to tag the *MALE STERILITY 2* gene in *A. thaliana* (Aarts et al., 1993), and a number of genes in maize (Walbot, 1992). Another transposon system from maize, comprising the nonautonomous Inhibitor or defective suppressor-mutator (I/dSpm) elements, was also used to tag and clone the *CES1* gene of *A. thaliana* (Aarts et al., 1995). The integration of *A. tumefaciens* transfer-DNA (T-DNA) during the transformation of *A. thaliana*, has also been used to generate hundreds of putative insertional mutants, from over 8000 independent transformational events (Feldmann and Marks, 1987; Feldmann, 1991), some of which have been characterised (He et al., 1996; Peirson et al., 1996).

Chemical mutagens, such as EMS, are alkylating agents which methylate DNA bases, causing mispairing and base pair transitions. The most common alkylation caused by EMS is the formation of O6-methylguanine, which mispairs with thymine, resulting in GC to AT transitions. Occasionally the formation of O4-ethylthymine may result in TA to GC transitions. Therefore EMS primarily results in point mutations, supported by sequencing data of mutagenised genes. Sequencing of two mutant *ADH* alleles, revealed one C to T transition and one G to A (Dolferus et al., 1990). The mutagenised rubisco activase gene (Orozco et al., 1993), and two mutant *TRP4* genes (Niyogi et al., 1993) were all found to contain a G to A transition. Mutations in three alleles of the *GA1* gene also showed single base pair transitions (Shirley et al., 1992). However, EMS does not only cause point mutations, and has been reported to cause deletions in maize (Okagaki et al., 1991), and chromosomal aberrations in *D. melanogaster* (Ashburner, 1989).
Ionising radiation causes a variety of DNA lesions, induced by the high linear energy transfer from ionising particles, which either interact directly with DNA, or indirectly via reactive species formed by the radiation energy. Fast neutrons have a higher linear energy transfer and densely ionised tracks, as compared to X and gamma rays which have a low energy transfer and sparse ionisations (Hawkins, 1979). Ionising radiation has no target specificity, and the major potential source for indirect damage to DNA is proposed by the reactive species formed by the radiolysis of water, predominant in living cells, and in particular the damage caused by hydroxyl radicals (Friedberg et al., 1995; Ward, 1975). The energy deposition from the track of a single charged particle of ionising radiation, occurs not as single ionisations, but in local clusters of ionisation along their tracks (Hutchinson, 1985; Sankaranarayanan, 1991; Simic et al, 1989; Ward 1988).

Since the deposition of radiation energy can cause local clusters of ionisations, more than two damaged moieties per "locally multiply damaged site" (LMDS), can occur in close proximity on the DNA. The distance separating LMDS is most significant over short distances, and can provide the conditions for the production of two double stranded breaks close together (Hutchinson, 1985; Sankaranarayanan, 1991; Simic et al, 1989; Ward 1988; Friedberg, 1995). Subsequently, DNA repair results in a variety of chromosomal aberrations including inversions, translocations, deficiencies and deletions (Koornneef et al., 1983; Sankaranarayanan, 1991; Shirley et al., 1992; Whitelam et al., 1993; Wilkinson and Crawford, 1991). Therefore ionising radiation predominantly causes chromosomal rearrangements, of which deletions are the most common (Sankaranarayanan, 1991), however a fast-neutron induced mutation of GA1, was found to contain a point mutation (Sun et al., 1992).

Two genes, involved in flavonoid biosynthesis, CHI (chalcone flavonone isomerase) and DFR (dihydroflavonol 4- reductase), were isolated from A. thaliana, using heterologous PCR primers. RFLP mapping indicated that the CHI gene mapped to chromosome III, in the region of the tt5 locus, and DFR mapped to chromosome V, in the region of tt3 (Shirley et al., 1992). The transparent testa, tt3 and tt5 mutations, were previously isolated using X-ray and fast-neutron irradiation respectively (Koornneef, 1990). Southern blot analysis of genomic DNA from tt3 and tt5, probed with DFR and CHI clones respectively, revealed that the DFR gene is completely missing in tt3, and that the CHI gene is inverted and contains a small deletion in tt5 (Shirley et al., 1992). Genomic clones isolated from tt5,
spanning the mutated \textit{CHI} locus, were analysed by restriction mapping and regions flanking the inversion site were sequenced. Analysis of the \textit{tt5} mutant clones, showed that the \textit{CHI} gene had incurred two break points, 1420 bp apart which had reinserted as an inversion, losing only 4 bp. Additionally a 272 bp fragment of DNA had also inserted into \textit{CHI} position in \textit{tt5} that was shown to have come from a site 38 cM distal from the \textit{CHI} gene, and was shown to be missing from its original position in \textit{tt5}, consistent with a translocation event (Shirley et al., 1992). Genomic clones from \textit{tt3}, showed that two deletions had occurred, one 7.4 kb deletion spanning the \textit{DFR} locus and another 52 bp deletion, 2.8 cM distal to the \textit{DFR} break. The intervening DNA had become inverted and the ends aberrantly rejoined, involving the inclusion of a 7 bp piece of filler DNA and short imperfect direct repeats (Shirley et al., 1992).

Two alleles of the \textit{GA1} locus, \textit{ga1-3} and \textit{ga1-2}, were isolated using fast neutron irradiation (Koornneef et al., 1983). One of the mutants, \textit{ga1-3}, a deletion mutant was used to isolate the \textit{GA1} gene, by genomic subtraction (Straus and Ausubel, 1990; Sun et al., 1992). Five cycles of subtractive hybridisation, were performed between sheared biotinylated DNA from mutant \textit{ga1-3} and Sau3A-digested nonbiotinylated DNA from from the WT parent ecotype \textit{L. erecta}. Remaining DNA fragments were ligated to Sau3A adaptors and cloned. One of the clones and clones subsequently isolated (the \textit{GA1} gene), mapped to the \textit{GA1} locus, complemented the \textit{ga1-3} mutant phenotype, and deduced the deletion in \textit{ga1-3} to be 5 kb in length (Sun et al., 1992). The isolation of the \textit{GA1} gene was also able to demonstrate that the \textit{ga1-2} allele contained a 3.4 kb fragment of DNA of unidentified origin being either an inversion or insertion (Sun et al., 1992).

\textit{A. thaliana} mutants with a lesion in the \textit{CHL3} gene, were generated using 30 krads of gamma-irradiation, on Columbia WT seeds (Wilkinson and Crawford, 1991). Mutagenised lines were selected for their resistance to chlorate, a nitrate analog converted by nitrate reductase to chlorite, which is toxic (Wilkinson and Crawford, 1991). Partial complementation of the \textit{chl3-5} mutant line was achieved with the nitrate reductase structural gene, \textit{NIA2}, which also indicated that the deletion in these mutants is at least 5 kb in length. Another deletion mutation was induced at the \textit{gl1} locus, involved in trichome formation. Two alleles, \textit{gl1-43} and \textit{gl1-1}, were induced using T-DNA insertional mutagenesis (Marks and Feldmann, 1989) and ionising radiation (Koornneef et al., 1982). Sequences from the T-DNA/GL1 border in \textit{gl1-43}, served as a probe to isolate an 8 kb DNA fragment
containing the uninterrupted \textit{GL1} locus (Herman and Marks, 1989). This fragment successfully complemented the \textit{gl1-1} phenotype, and was used to isolate the \textit{gl1-1} genomic clone. Restriction enzyme mapping of the \textit{gl1-1} clone, revealed that the deletion in \textit{gl1-1} was 6.5 kb (Oppenheimer et al., 1991).

One advantage of deletion mutants, is that they provide a null background, useful for complementation analysis, avoiding the effects of cosuppression, a phenomenon whereby unknown interactions occur between a transformed gene and the corresponding endogenous gene, causing a type of gene silencing, as reported for a chimaeric chalcone synthase gene (Napoli et al., 1990). However, a null genotype generated by deletion, or indeed insertional mutagenesis, means that mutations in essential genes, may lead to non-viability, and the inability to recover such mutations. The induction of point mutations in essential genes, may circumvent this problem, by causing resistant or leaky alleles which are recoverable, as seen for mutant alleles of the \textit{csr} gene (Haughn and Sommerville, 1988; Mourad et al., 1994).

The mutations arising within a mutagenised population can be manifested in either the phenotype or genotype of the organism. In \textit{A. thaliana} phenotypic mutants demonstrate abnormal growth or structural defects, like \textit{eceriferum} mutants with aberrant surface wax layers, or mutants with abnormal flower, leaf or trichome morphology. Phenotypic mutants are readily identified from a M2 population by visual screening. However, where the mutation results in a phenotypically normal plant, screening of the M2 population requires an alternative strategy, which can be based around the biochemical or genetic consequence of the mutation.

Biochemical lesions that confer resistance to toxic compounds, have been used to isolate mutant \textit{A. thaliana} lines resistant to the herbicide chlorosulfuron (Haughn and Somerville, unpublished) or the growth inhibitors coronatine or methyljasmonate (Feys et al., 1994). Chlorosulfuron resistant lines were selected by screening 300,000 M2 seeds on agar medium containing the herbicide (Haughn and Somerville, unpublished), and the mutants isolated were found to have altered acetolactate synthase activity, conferring chlorosulfuron resistance. The \textit{A. thaliana} mutant lines which grew on 1 \textit{M} coronatine, were all found to be affected at a single locus, \textit{coi1} (Feys et al., 1994). Mutants resistant to abscisic acid (Koomneef et al., 1984; Finkelstein and Somerville, 1990), auxin (Maher and Martindale, 1980; Estelle and Somerville, 1987; Wilson et al., 1990), ethylene (Bleecker et al., 1988;
Guzman and Ecker, 1990) and gibberellin (Koornneef et al., 1985) have also been isolated.

Similar strategies have been used to isolate A. thaliana lines, which because of their mutation are unable to convert a non-toxic substrate to a toxic one, allowing the direct selection of such mutants. This approach was used to isolate mutagenised lines of A. thaliana resistant to the exogenous application of chlorate or 2,6-diaminopurine (DAP). These substrates are converted by endogenous plant enzymes to the toxic compounds chlorite and a nucleotide form of DAP respectively, leading to the isolation of mutants for the nitrate reductase and adenine phosphoribosyl transferase genes respectively (Oostinder-Braaksma and Feenstra, 1973; Wilkinson and Crawford, 1991; Moffatt and Somerville, 1988).

Other tactics for the isolation of mutant lines, have involved direct biochemical or genetic assays, examining individual plants within M2 families. Although such approaches may be time consuming, they have identified a number of mutant A. thaliana lines. Gas chromatography was used to isolate mutants with altered membrane fatty acid composition (Browse et al., 1985), and the staining of leaf material with iodine was able to identify mutants unable to synthesise starch (Casper et al., 1985). The exposure of mutagenised populations to different wavelengths of light has identified mutants defective in light perception (Parks et al., 1989; Koornneef et al., 1980; Chory et al., 1989; Whitelam et al., 1993) some of which were mutagenised in one of the phytochrome photoreceptors. Embryo lethal mutations have been isolated by scoring the progeny of heterozygous lines for segregating lethal mutations (Meinke, 1985).

In this work, a transgenic M2 population was assayed for the down regulation of specific promoters, in order to identify A. thaliana mutants affected in stamen development. Genetic screening techniques have been used by other research groups (Takahashi et al., 1992; Jackson et al., 1995), which utilised relevant promoter fusions to the GUS gene. A. thaliana seed, transgenic for the promoter-reporter fusions were mutagenised, and mutants showing an altered pattern of GUS expression analysed. Takahashi and coworkers used the heat shock promoter, HSP18.2, to screen for mutations in genes involved in the induction of heat shock protein genes, and isolated three lines, hs2, hs-5 and hs7, that reproducibly exhibited a reduction in heat-inducible GUS activity. Jackson and coworkers, employed the chalcone synthase (CHS) gene promoter, to screen for mutants showing altered light induction of the transgene. Two mutants, A12 and
C10, showed elevated levels of GUS activity in the light, which corresponded to an increase in CHS transcript levels.

The LUC (Ow et al., 1986) and TMS2 (Karlin-Neumann et al., 1991) genes, have also been proposed for use in transgenic screening. The use of the cab140 phytochrome promoter, fused to the TMS2 gene, transformed into A. thaliana prior to mutagenesis, has been proposed as a viable stratagem for positively screening for putative mutants unable to respond to red light (Karlin-Neumann et al., 1991). Putative mutants germinated in red light would grow on a α-NAM and β-NAM mixture, inactive auxin substrates of the TMS2 gene, while selecting against WT seedlings whose growth is inhibited by the active toxic auxins produced as a consequence of normal cab140, and thus TMS2 gene expression. Photon imaging of transgenic M2 A. thaliana, expressing the LUC gene, under the control of the CAB2 promoter, has also been employed to screen for mutants with aberrant cycling patterns (Millar et al., 1995).

Once isolated, the characterisation of a new A. thaliana mutant line should start with a phenotypic analysis, and the performance of allelic test crosses with known mutants of a similar phenotype. The genetic analysis of the new line should involve the determination of inheritance and physical mapping of the mutation. The monogenic, digenic or polygenic inheritance of a new mutant phenotype can be determined by analysing the segregation of the mutant phenotype through subsequent generations, and also establishes whether the mutation is dominant or recessive. The performance of a backcross to WT and subsequent progeny analysis, and the co-segregation analysis of the mutant with other tester lines can also help to establish the nature of inheritance of a new mutant line.

The physical mapping of a new mutation to one of the five chromosomes, may be achieved by determining the linkage of the mutation to existing classical or molecular markers, already placed on the integrated physical and genetic map of A. thaliana. Linkage analysis allows the genetic distance of the mutation from a particular marker to be calculated, and depends on the recombination frequency occurring between the mutation and the marker within a segregating population.

A preliminary map position of the new mutation can be achieved by crossing to the multi-marker tester line W100 or W100F, which carries one or two visible markers on each chromosome. Such a cross between a mutant and marker line, generates a heterozygous F1 plant in which the mutation and markers are in repulsion phase (R). Phenotypic analysis of the resulting F2(R) population allows
the detection of linkage between the mutation and one of the markers, enabling the mutation to be assigned to one of the chromosomes. Crossing a mutant F2(R) plant carrying the new mutation and one or more of the visible markers, to WT generates coupling-phase heterozygous plants (C). These F1(C) plants can then either be selfed to generate F2(C) plants or crossed back to the original W100 or W100F marker line, to generate test cross progeny (TC). The detection of linkage in F2(C) and TC plants requires fewer plants to detect linkage and gives a more accurate map position for the new mutation.

Once a rough position has been obtained, fine mapping of a new mutation can be achieved using mapped molecular markers, such as restriction fragment length polymorphisms (RFLPs) (Chang et al., 1988; Nam et al., 1989), Random amplified polymorphic DNA (RAPDs) (Reiter et al., 1992), co-dominant cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel, 1993), simple sequence length polymorphisms (SSLPs) (Bell and Ecker, 1994) or the RI lines (Lister and Dean, 1993). Such DNA mapping techniques can be carried out on F2 or later segregating populations (C or R phase) and depend on the existence of polymorphisms between two ecotypes at a single locus, enabling the recombination frequencies to be calculated for the mutation and the locus under investigation. Recombination frequencies can be used to calculate the recombinant fraction \( p \), by the product ratio (PR) method, which is the product of the two nonparental phenotypic classes divided by the product of the two parental classes. The PR relates to \( p \), and can be found listed in tables (Stevens, 1939). Using mathematical formulae known as mapping-functions (Haldane, 1919; Kosambi, 1944) the genetic distance, expressed in cM, can be calculated from the \( p \) value.

The subsequent isolation of a mutagenised gene by map based cloning, utilising the technique of chromosome walking, relies on the availability of an accurate map position of the new mutation, and the availability of a cloned DNA sequence that maps close to the mutation. The nearby cloned DNA sequence is used as a probe to isolate the corresponding clone from a WT *A. thaliana* genomic library. This clone is used in turn to isolate successive overlapping genomic clones, until a clone containing the gene mutagenised in the mutant is isolated. The isolation of the correct genomic clone is confirmed by the transformation of the mutant with the genomic fragment, resulting in the complementation of the mutant phenotype.

In this work, the transgenic screening (5.1) of a \( \gamma \)-irradiated M2 population
was adopted to isolate mutants of *A. thaliana*, affected in stamen development. Homozygous lines of *A. thaliana*, transgenic for either the A6 (Hird et al., 1993) or A9 (Paul et al., 1992) tapetal-specific promoter fused to the *GUS* gene were isolated from a number of supplied transgenic lines. Homozygous A6-*GUS* and A9-*GUS* transgenic seed were mutagenised using Caesium\(^{137}\) (Cs\(^{137}\)), a γ-irradiation source, and the resulting M1 plants were allowed to self-fertilise. M2 families were assayed for the down regulation of GUS activity in whole whorls of flower buds. Putative mutant lines, showing a loss or reduction of GUS activity, implied a lesion upstream of A6 or A9 expression, during the early stages of microsporogenesis and such lines were selected for further genetic and phenotypic investigation.

6.2 Isolation of homozygous A6-*GUS* and A9-*GUS* transgenic
*A. thaliana* lines

A number of *A. thaliana*, T2 seed lines, transformed with either A6 or A9-*GUS* were supplied by Biocem (France). Segregation analysis was carried out on six individual T3 generations from both A6 and A9-*GUS* transgenic lines, by assaying flower buds for GUS activity using X-GLUC staining and fluorimetric detection of the GUS gene. Two lines, A6-17 and A9-127, were selected for their 3:1 segregation of the transgene in the progeny of hemizygous plants (Tab. 6.1). The segregation of these T3 seedlings on kanamycin, also showed the expected pattern of 3:1 for kanamycin resistance: kanamycin sensitivity (Tab. 6.1), indicating the monogenic inheritance of each transgene. A T3 individual expressing the *GUS* gene was selected for each transgenic line, and T4 seeds were harvested and stored. Over 100 T4 A6-17 and A9-127 seeds were germinated on MS medium containing kanamycin. Both chosen lines showed 100% resistance to kanamycin indicating the homozygous nature of each transgene. Bulked T4 seeds of A6-17 and A9-127, were subsequently used for γ-irradiation dose trials and for the following large scale mutagenesis experiment.

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6.3 Generation of a mutagenised \textit{A. thaliana} population

6.3.1 \textgamma-Irradiation dosage experiment

Since ionising radiation is known to induce mutations at a frequency dependent on a number of different factors, including the nature of the target material, and in particular the moisture content of the seed, small scale dose trials was carried out on a small number of transgenic seed. In doing so, an optimum dose of radiation could be determined, which was sufficient to induce a high frequency of mutagenesis, while not being significantly detrimental to the viability of the resulting population. The ionising radiation used was a gamma irradiating Cs\textsuperscript{137} source which emitted 0.923 Krads/min. To evaluate the optimum dose, a small number of A9-\textit{GUS} transgenic seed were exposed to the source for 32.5, 65, 97.5 or 130 minutes. The irradiated seeds were sown onto moist soil, covered and left overnight at 4°C, after which time they were transferred to the greenhouse. The resulting seedlings, (Fig. 6.1) showed that the seeds irradiated for 130, 97.5 and 65 minutes showed a poor germination rate and those that did germinate soon died. These seedlings were also much smaller than non-irradiated, transgenic seedlings sown at the same time. In contrast, seeds irradiated for 32.5 minutes showed as good a germination rate as non-irradiated A9-\textit{GUS} plants and the resulting plants were only slightly smaller in stature. These observations indicated an ideal seed exposure time of 32.5 minutes (30 Krads of Cs\textsuperscript{137}), to generate the large mutagenised population of \textit{A. thaliana}, suitable for screening for mutants without being detrimental to the viability of the population.

6.3.2 Large scale irradiation of transgenic seed

Approximately 4 000 seeds from each A6-17 and A9-127, homozygous monogenic line, were placed in separate 1.5 ml microfuge tubes. The seeds were exposed to 30 Krads of Cs\textsuperscript{137}, and on the same day approximately 2 000 M1 seed from each line were sown onto a moist 3:1 compost/sand mixture. The remaining seed was stored at -80°C. The M1 seeds were sown at a rough density of 200 seeds/tray (approximately equivalent to 1 seed/4 cm\textsuperscript{2}). The trays were covered with clingfilm and stored o/n at 4°C, after which time they were transferred to the greenhouse. Once the first true leaves were formed the clingfilm was removed from the trays and the seedlings were grown to maturity. M1 plants were allowed to flower and self-pollinate.
6.3.3 Generation of M2 mutant population

Individual, dried siliques were harvested from each M1 plant and stored in separate 0.5 ml microfuge tubes. At weekly intervals, 12 trays containing a 3:1 compost/sand mix were sown with mutagenised seed. Each tray consisted of 24 separate plastic compartments, each of which was sown with 10 M2 seeds from a single M2 family, to ensure the detection of recessive mutations. Trays were covered with clingfilm and stored at 4°C for 5 days, and then transferred to the greenhouse. Once the seeds had germinated, the clingfilm was pierced with holes to reduce the humidity, and removed a further 3 days later. M2 plants were allowed to continue development until floral inflorescences were produced. Each M2 family member was then screened for recessive mutations, by assaying for a down regulation of GUS activity in the flower buds.

6.4 Numbers of M2 plants screened and mutation frequency

A total of 2 866 M2 A. thaliana families were screened for GUS activity, of which 1 241 families were A6-GUS transgenic M2 A. thaliana lines, and 1 625 were A9-GUS (Tab. 6.2). The microtitre plate screening involved 25 375 individual GUS assays, and therefore on average 8.85 plants were screened per M2 family. The probability (P) of recovering a recessive mutation, is:

\[ P = 1 - f^n \]

whereby, f is the fraction of families that would have no mutation and n is the number of plants in a M2 family. Therefore, from an average of 8.85 plants screened / M2 family there is a 92.2 % probability of recovering at least 1 homozygous recessive individual.

Approximately 1.5 % of the M2 population, showed by segregation to carry a detectable recessive mutation, a number of which had been previously characterised (6.4.3). Several chlorophyll (Fig. 6.2.a), or growth inhibited (Fig. 6.2.b) mutants were visualised, including one variegated line and a few albino lines. A similar number of embryo-lethal mutants were also scored, demonstrated by the presence of black inviable seeds in the siliques (Fig. 6.2.c). One M2 family, produced homozygous mutants showing reduced stature, resulting in a tightly compacted dwarf plant with faciated stems, designated cactus. (Fig. 6.2.d). A further 0.24 % of the M2 population (7 individual families) showed floral mutations; 1.08 % (31 families) exhibited male-sterility, and 0.27 % (8 families) showed down regulation of the GUS gene in the flower buds, of which only 0.07 % (2 families).
were male-fertile.

6.4.1 Previously characterised mutants

A number of the phenotypic mutants isolated showed identical phenotypes to previously characterised mutants. Of the chlorophyll mutants, isolated in this screen, an albino line demonstrated an elongated hypocotyl (Fig. 6.2.e), and was a phenocopy of either the HY1 or HY2 mutant (Koornneef et al., 1980; Parks et al., 1989; Parks and Quail, 1991), which are phenotypically indistinguishable. Of the floral mutants visualised in this screen, phenocopies for the apetala mutants, ap1 (Fig. 6.2.f), ap2 (Fig. 6.2.g) and ap3 were identified (1.1.1), as well as the pin-formed mutant (Fig. 6.2.h; Goto et al., 1991). The mutant line designated cactus (Fig. 6.3.d), showed a similar phenotype to two mutants documented on the A. thaliana Nottingham database, stock numbers: N425 and N371, although complementation analysis has not been carried out to test allelism.

6.4.2 Screening for mutants affected in anther development

Mutant lines were identified from the M2 population, as plants that showed a down regulation of the GUS gene in the developing floral buds. GUS assays were carried out in transparent microtitre plate wells, and GUS activity was detected fluorometrically and determined visually, using ultra-violet illumination. The microtitre plates used consisted of 8 rows of 12 wells, labelled A to H. Consequently M2 trays were divided into 3 sections of 8 compartments, each consecutively marked with A to H, so that one row of wells from the microtitre plate, corresponded to a single labelled M2 family. Therefore the loss or reduction of GUS activity detected in one of the wells, would enable the isolation of the mutant family responsible.

6.4.3 Microtitre plate GUS assays

To aid the screening process, a quick and easy GUS assay detection system was devised. A single whorl of flower buds was taken from each individual M2 plant and ground in a microtitre plate well. Each well contained a GUS assay phosphate buffer and 1 mM MUG, the GUS substrate. Microtitre plates were incubated at 37°C for at least 2 hours. Positive GUS activity was visualised as fluorescence, produced by 4-MU, the product of the GUS enzyme, under ultra-violet illumination (Fig. 6.3).
6.4.4 GUS negative lines

Mutant lines identified, showing a down regulation of the GUS gene, were defined by the prefix “gne” and numbered consecutively on their isolation. Screening the A6-GUS M2 population for the down regulation of GUS activity identified two mutant lines, designated gne3 and gne4, both of which were ms. GUS assays carried out on the A9-GUS M2 population revealed 6 mutant GUS negative lines, namely gne1, gne2, gne5, gne6, gne7 and gne8. Four of the lines, gne1, gne2, gne5 and gne7 were ms, while gne6 and gne8 were male fertile. All homozygous mutant lines showed a complete loss of GUS activity, except gne7, which showed 1 to 2 % GUS activity, as compared to buds from unmutated A9-GUS transgenic plants. The absence or reduction in GUS activity was confirmed in the M3 progeny of all eight lines, and was found to cosegregate with the ms condition in the six relevant lines. In the M3 progeny from both fertile GUS negative lines, the presence of at least part of the GUS gene was demonstrated by PCR analysis. All GUS negative mutant lines were found to be fully female fertile, except gne7 which shows reduced female fertility.

6.5 Characterisation of mutant GUS negative lines

6.5.1 Testing for the integrity of the transgene

To determine if the two male fertile, GUS negative mutants were a genuine in trans down-regulation of the A9-GUS transgene, both mutant lines gne6 and gne8, were crossed to the multi-marker line W100F. Flower buds from the F1 progeny were assayed for GUS activity and in both cases no GUS activity was detected, indicating that the lesion caused by the γ-irradiation had occurred within the transgene, either in the promoter or the GUS gene. No further analysis was carried out on these lines. All ms, GUS negative lines were similarly outcrossed to W100F and all F1 progeny showed GUS activity in the flower buds, indicating a genuine trans down regulation of the GUS gene. Therefore further investigative analysis was carried out on ms, GUS negative lines only.

6.5.2 Temporal comparison of lesions in GUS negative mutants with 3 existing ms mutants

To investigate if the transgenic screening strategy adopted, had the potential to isolate mutants which carry lesions that act earlier in sporogenesis than three existing sporogenesis mutants, promoter-GUS transgenes were introduced by
crossing non-mutated, homozygous A6-17 or A9-127 plants into two meiotic ms developmental mutants, \textit{ms32} and \textit{ms37} (1.4.2.1), and one post-meiotic mutant \textit{ms33} (1.4.2.2), courtesy of Dr. Bernard Mulligan at the Nottingham \textit{Arabidopsis} stock centre. F2 progeny which were ms and carried the promoter-GUS transgene, demonstrated by PCR analysis using primers designed against the GUS gene, were assayed for GUS activity in the flower buds to establish if these plants would still express the GUS transgene. GUS activity was detected in the flower buds of all ms transgenic F2 plants tested (Tab. 6.3). This demonstrates that the GUS negative mutants isolated carry lesions, disrupting microsporogenesis, that act earlier than the mutations present in \textit{ms32}, \textit{ms33} and \textit{ms37}. From over 300 \textit{ms37}/A9-127 F2 plants grown, no ms individual gave a positive PCR result for the GUS gene, indicating the tight linkage between the transgene (A9-GUS) and the \textit{ms37} mutation. These results indicate the strategy adopted, utilising the A6 and A9 promoters in transgenic screening approach, would not have been able to isolate mutants such as \textit{ms32}, \textit{ms33} and \textit{ms37}. Therefore use of the A6 and A9 promoters provides a mechanism to look for mutants along the same pathway of stamen development in a different temporal region.

6.5.3 Observations on mutant phenotypes and floral morphology

In five of the six GUS negative lines, \textit{gne1}, \textit{gne2}, \textit{gne3}, \textit{gne4} and \textit{gne5}, the lesion(s) present had no visible effect on vegetative development in homozygous plants. Floral morphology in these homozygous mutant lines, including the initial formation of the stamens, was also comparable to WT. However, the mutations harboured in all five lines, caused a disruption in anther development leading to a failure in pollen grain production and male sterility. Consequently fertile stigmas remained unpollinated, resulting in short siliques devoid of seed.

Plants homozygous for the \textit{gne7} mutation were also ms, although a few pollen grains were produced by some anthers. Additionally \textit{gne7} homozygous plants were smaller in stature than WT plants (Fig. 6.4.a; 6.4.c), and flowers showed varied and abnormal numbers of petals, stamens or carpels (Tab. 6.4, Fig. 6.4.e). Stamen morphology in these lines was also aberrant, and anthers developed three abnormally shaped lobes (Fig. 6.4.g) instead of two symmetrical theca (Fig. 6.4.f).
5.5.4 Observations on mutant anther histology

5.5.4.1 Analysis of anther transverse sections using light microscopy

To investigate the abnormal programme of microsporogenesis that results in male sterility in all six GUS negative mutants identified, a sequential developmental series of anther transverse sections were examined for each mutant line and compared to WT. The process of pollen formation during WT *N. tabacum* and *B. napus* development, has been demonstrated to follow a tightly regulated series of events, involving the synchronous differentiation of the four locules of the anther, which can be delimited by cytological landmark events, that show a direct correlation to bud or anther length (Koltunow et al., 1990; Scott et al., 1991a; Scott et al., 1991b). In order to establish this temporal relationship in *A. thaliana*, WT buds form the C24 ecotype were harvested, measured along their lengths and transverse sections through the anthers were analysed for their developmental stage using toluidine blue staining and light microscopy (5.2).

6.5.4.1.1 Microsporogenesis in WT *A. thaliana*

Light micrographs of anther transverse sections from C24 (Fig. 6.5.a-f), show the main stages of early WT development, from diploid meiocytes to haploid uninucleate microspores. In a bud length of 0.39 mm (Fig. 6.5.a), the anthers contain premeiotic meiocytes (premeiosis I), which are tightly packed together and have an angular shape. The central core of diploid meiocytes are closely enveloped by the four continuous, concentrically arranged, single cell layers of the anther wall. The tapetum is innermost lying adjacent to the developing meiocytes, then the middle layer, the endothecium and the outermost epidermis. At this stage of development the tapetal cells are relatively small, have a dense cytoplasm and only one or two small vacuoles.

Later during premeiosis I, at a bud length of 0.44 mm (Fig. 6.5.b) the tapetal cells have become larger, square shaped cells. At a bud length of 0.49 mm (Fig.6.5.c), separation has occurred between the microsporocyte wall and the cytoplasm of each meiocyte (premeiosis II), although the meiocytes still occupy the entire locular space. At this stage of development, the tapetal cells are fully differentiated, compact vacuolate cells. As development proceeds the meiocytes become more rounded, enter meiosis and synthesise a complete callose coat.

Subsequently, cytokinesis and accompanied callose deposition along the newly synthesised cell wall, results in the formation of tetrads of four haploid...
microspores, each encased and held together within the callose coat (Fig. 6.5.d). At this stage, with a bud length of 0.56 mm, the tapetal cells are large, separate cells that contain a large vacuole, while the middle layer has become squashed and flattened. At an approximate bud length of 0.69 mm, the callose coat is degraded and the microspores are released into the locular space (Fig. 6.5.e), the tapetal cells have started to degenerate and are losing their cell shape, and the middle layer by this stage is completely absent. At around a bud length of 0.7 mm (Fig. 6.5.f), an exine coat surrounds the microspores, which stains darkly with toluidine blue, and the microspores become increasingly vacuolate pushing the nucleus to one side of cell (in preparation for mitosis, which gives rise to the generative and vegetative cells). At this stage of development the tapetal cells stain very faintly, indicating their continued and rapid deterioration, which results in their complete degeneration.

In order to establish a link between the ms phenotype, seen in all six GUS negative mutants, and any visible lesion occurring within the anther, a similar series of buds were harvested from each mutant line, and examined for their progression through microsporogenesis. Examination of these sequential anther transverse sections showed that five of the six mutants fell into two phenotypic mutant classes. In the first class, \textit{gne1}-like, comprising \textit{gne1} and \textit{gne4}, the normal differentiation of the two innermost sporangia cell types, the tapetum and the middle layer, are affected. In the second mutant class, \textit{gne2}-like, comprising \textit{gne2}, \textit{gne3} and \textit{gne5}, the continuity of the tapetum and middle layer is disrupted and the number of sporogenous cells is abnormally high. While in the sixth mutant \textit{gne7}, a third abnormal course of microsporogenesis is seen in the sporogenous cells, which demonstrate an asynchronous pattern of development.

\textbf{6.5.4.1.2 Microsporogenesis in gne1 mutant class}

In the \textit{gne1} mutant class, consisting of \textit{gne1} (Fig. 6.8.a-h) and \textit{gne4} (Fig. 6.8.i-l), examination of the earliest anther transverse sections, reveals that the initial development of the stamen, resulting in the four differentiated microsporangia of the anther, occurs as in WT development. At this time in \textit{gne1}, during premeiosis I at a bud length of 0.35 mm (equivalent \textit{gne4} stage not shown), the four cell layers of the microsporangium are clearly visible as single cell layers, concentrically arranged around the central core of morphologically typical meiocytes (Fig. 6.6.a). Here as in WT development, the middle layer cells of \textit{gne1} are small,
comparatively elongated cells, with respect to the other three cell types that make up the anther wall at this stage. The tapetal cells also appear normal, and are shorter, more compact cells, and like their WT counterparts contain only one or two small vacuoles.

Following this apparently normal initiation of microsporogenesis, during a stage which still appears to be premeiosis I (no separation between the meiocyte wall and cytoplasm), *gne1* with a bud length of 0.36 mm (Fig. 6.6.b) and *gne4* (Fig. 6.6.i) with a bud length of 0.49 mm, both possess tapetal cells that have increased in size in line with WT development. However, already a slight increase in the vacuolation, compared to WT, of some of the tapetal and one or two of the middle layer cells can be seen. The development of the meiocytes, at this time, appears to remain unaffected. At a later stage in *gne1* (Fig. 6.6.c) and *gne4* (Fig. 6.6.i) development, at bud lengths 0.46 and 0.54 mm respectively, a further increase in size and vacuolation of some of the tapetal and middle layer cells is apparent. Additionally, there is no separation of the meiocyte cell wall from the cytoplasm, as in premeiosis II, which is observed during the normal progression of microsporogenesis at around this time. This may be a consequence of the enlarged tapetal cells encroaching on the locular space.

At a bud length of 0.51 mm in *gne1* (Fig. 6.6.d), and 0.60 mm in *gne4* (Fig. 6.6.k), the tapetal and middle layer cells have increased in size and started to encroach upon the locular space ordinarily occupied by the meiocytes, impeding their development. The tapetal and middle layer cells possess very little cytoplasm as the majority of the cell contains a grossly enlarged, abnormal vacuole. The meiocytes enter meiosis as confirmed by the production of callose (initially normally detected during prophase I: Scott et al., 1991a) which fills any locular space, not taken up by the still tightly compacted meiocytes. A comparative longitudinal section through an anther from *gne1*, of bud length 0.44 mm (Fig. 6.6.e), illustrates both the gross enlargement and vacuolation seen in both the tapetal, and to a lesser extent the middle layer cells, and the production of callose during the initial stages of meiosis. By a bud length of 0.75 mm in *gne1* (Fig. 6.6.f), and as early as 0.6 mm in *gne4* (Fig. 6.6.l), both the tapetal and middle layer cells have become so grossly enlarged as to cause the tetrad of microspores now present in the locule to become distorted. Continued enlargement of the vacuolated tapetal cells is seen in this mutant class, and in *gne1*, by a bud length of 0.89 mm (Fig. 6.6.g), the tapetal cells have completely invaded the locular space crushing the tetrads. The remnants
of the tapetal cells and tetrads completely degenerate by a bud length of 0.95 mm leaving an empty locule within the deflated, pre-dehiscent anther (Fig. 6.6.h).

### 6.5.4.1.3 Microsporogenesis in gne2 mutant class

In the gne2 mutant class, consisting of gne2 (Fig. 6.7.a-i; Fig. 6.9.a-b), gne3 (Fig. 6.8.a-f) and gne5 (Fig. 6.8.g-l; Fig. 6.9.c-d), anther transverse sections show that the histology of the microsporangium is aberrant from an early stage. In gne2, at a bud length of 0.35 mm (Fig. 6.7.a), the anther wall consists of only two or three cell layers, with only the two outermost cell types, the presumptive epidermis and endothecium, forming continuous layers. In addition, the morphologically normal meiocytes present within the anther locule, are more numerous than those found in a WT anther of a similar developmental stage. In another anther from gne2, taken from a 0.43 mm long bud (Fig. 6.7.b), the continuity of the microsporangial wall is also disrupted, although in places four cell layers are visible. In a transverse section through gne2 (Fig. 6.7.c), at a bud length of 0.47 mm, only two or three cell layers of the sporangial wall are visible. Where only two cell layers are present, the sporogenous cells lie adjacent to endothecium cells. While in gne5 (Fig. 6.8.g), at a bud length of 0.48 mm isolated presumptive tapetal cells are visible. In a comparative, longitudinal section from gne3 (Fig. 6.8.a), at bud length of 0.49 mm, it can be seen that the tapetal and middle layer cells of this mutant class, do not form a continuous layer around the meiocytes as is found in WT. Instead, isolated islands of middle and tapetal like cells are formed. In some sections of the anther transverse section, the normal configuration of four cell layers is found in the microsporangium and in other places the meiocytes are seen developing adjacent to endothecium cells. Additionally this longitudinal section of gne3, highlights that the number of sporogenous cells, in this mutant class, is abnormally high.

In gne2 at a bud length of 0.53 mm (Fig. 6.7.d and Fig. 6.7.e), and gne5 at a bud length of 0.59 mm (Fig. 6.8.h) , anther histology and meiocyte morphology remain unchanged, except for an increase in vacuolation of the tapetal cells. The meiocytes remain tightly packed in the anther locule, showing no separation between their cytoplasm and cell wall. As development proceeds in gne2 (Fig. 6.7 f and Fig. 6.7.g), gne3 (Fig. 6.8.b) and gne5 (Fig. 6.8.i and Fig. 6.8.j), at bud lengths 0.57, 0.55, 0.63 and 0.60 mm respectively, the meiocytes start to secrete callose. In some areas of these anther transverse sections, the microsporangium wall is three cell layers deep, and a single cell layer which may be middle layer or inner
secondary parietal cells is observed between the meiocytes and the endothecium (Fig. 6.7.g).

As callose deposition continues in gne2 (Fig. 6.7.h), gne3 (Fig. 6.8.c and Fig. 6.8.d) and gne5 (Fig. 6.8.k), at bud lengths 0.48, 0.55, 0.64 and 0.57 mm respectively, some of the meiocytes have become separated. The islands of tapetal and middle layer cells are still present, and the tapetal cells have enlarged and contain a large vacuole that occupies the majority of the cell. In gne2 (Fig. 6.7.i) and gne3 (Fig. 6.8.e), at bud lengths 0.69 and 0.58 mm respectively, the vacuolate tapetal cells have grossly enlarged. The meiocytes have become distorted. Some of the meiocytes have undergone meiosis to form distorted meiotic products. In gne2 (Fig. 6.7.j), gne3 (Fig. 6.8.f) and gne5 (Fig. 6.8.l), at bud lengths 0.75, 0.58 and 0.81 mm, in gne2 (Fig. 6.7.k) the products of meiosis have started to degenerate. By a bud length of 0.80 mm, the gynoecium (Fig. 6.10.a-t) shows for each equivalent stage of pollen development.

In gne7 (Fig. 6.10.a), at a bud length of 0.35 mm, the anther wall and the sporogenous cells of the microsporangium are not fully differentiated. At this stage, the gynoecium (Fig. 6.10.b) has not initiated development of the ovules. At bud length of 0.5 mm (Fig. 6.10.c), premeiotic meiocytes are surrounded by the four anther cell walls, the tapetum, the middle layer, the endothecium, and the epidermis. The corresponding gynoecium Fig. 6.10.d) shows that the ovule primordia have emerged as a dome of cells. At a bud length of 0.65 mm, the meiocytes (Fig. 6.10.e), are undergoing meiosis and have secreted a complete callose wall. Tapetal cells are large, separate, vacuolate cells, the middle layer has become flattened. In the gynoecium (Fig. 6.10.f), the ovules have synchronously
increased in length and width.

In one anther of gne7, taken from a 0.76 mm long bud, one locule contains a premeiotic microspore (Fig. 6.10.g), surrounded by an intact tapetal and middle cell layer, while an adjacent sporangium contains a post meiotic tetrad (Fig. 6.10.h), surrounded by degenerating tapetal cells, and one or two remnants from the middle layer. The gynoecium of this anther (Fig. 6.10.i), shows the two developing ovules extending in unison. In a bud of 0.95 mm in length, the asynchronous pattern of pollen development can be seen between adjacent anthers. In one anther locule (Fig. 6.10.j), tetrads and highly vacuolate tapetal cells are present, whilst an adjacent locule from another anther (Fig. 6.10.k), has early microspores and degenerating tapetal cells at its periphery. A third locule (Fig. 6.10.l) contains prematurely degenerating microspores, where the plasma membrane enclosing the cytoplasm has shrunk away from the exine wall; here the tapetal cells are irregularly shaped and show further signs of disintegration. In the gynoecium from this bud (Fig. 6.10.m), the ovules have grown in length and width.

In a larger bud length of 1.08 mm, one locule contains early microspores with an exine coat, where the cytoplasm and plasma membrane are still in contact (Fig. 6.10.n), while another locule shows degenerate microspores at a later stage where the exine wall and plasma membrane have dissociated (Fig. 6.10.o). Gynoecium development at this stage (Fig. 6.10.p), indicates progressive enlargement of the ovules. By a bud length of 1.27 mm, all the anther locules contain exine coated microspores, some of which have plasma membranes still in contact with the exine wall (Fig. 6.10.q), while others show dissociation and degeneration of the cytoplasm, resulting in pollen abortion (Fig. 6.10.r). In all locules the tapetal cells are degenerating. At this stage the ovules of the gynoecium have simultaneously increased in length and circumference (Fig. 6.10.s).

6.5.4.2 Analysis of anther transverse sections using T.E.M.

To determine if the lesions observed in the tapetal or middle layer cells of gne4 could be related to abnormalities in cell composition, sized mutant buds from both gne4 and WT C24 were embedded in Spurr resin, and sections were examined using transmission electron microscopy (T.E.M.) Anther transverse sections from gne4 revealed the tapetal cells possess a less dense cytoplasm, with a lower ribosome density and a reduction in endoplasmic reticulum and other functional bodies than compared to WT counterparts (Fig. 6.11).
6.5.5 Allelism test crosses between GUS negative mutants

Due to the similarities observed in gne1- like and gne2- like mutants, allelism test crosses were carried out within the two groups. gne1 was crossed to a gne4/GNE4 F1 plant; gne2 was crossed to a gne3/GNE3 F1 and to an F1 from a gne5/pAF11 cross (pAF11 is a C24 ecotype of A. thaliana transformed with an A6-GUS/A9-LUC, chapter 5) and gne3 was crossed to an F1 resulting from a gne5/pAF11 crossing. The number of F1 ms resulting from these crosses was scored (Tab. 6.5). The presence of ms plants occurring in the F1 progeny indicates allelism between the two mutations used in the crossings. Therefore this data shows that gne2, gne3 and gne5 are allelic and that gne1 and gne4 are not.

6.6 Mapping mutations to chromosomal regions

Through the determination of recombination occurring between a mutation and a visual (W100F) or genetic (CAPS) marker, the distance of the mutation in cM, from the marker can be calculated. Utilising the Integrated maps of A. thaliana (Hauge et al., 1993), which gives map positions for a number of classical and RFLP markers, the approximate map position of the new mutation can be ascertained. The Integrated maps of A. thaliana (Hauge et al., 1993), were obtained by the integration of three previously independent maps (Chang et al., 1988; Nam et al., 1989; Koornneef et al., 1990). The order of the markers placed on the Integrated maps, were generated using the fixed order option of JOINMAP (Stam, 1993).

6.6.1 Mapping using the multimarker tester line W100F

In order to assign the GUS negative mutations on the A. thaliana genetic map, the multimarker line W100F was crossed to each mutant line. W100F (Koorneef et al., 1987 and 1992) carries 9 recessive genetic mutations, spread over the five A. thaliana chromosomes (Tab. 6.6), each of which displays an individual phenotype (Tab. 6.6). Visual analysis of the cosegregation of the 9 mutations from W100F and ms from each mutant, in a large individual F2 population, allows the determination of linkage to be established between one of the markers from the W100F tester line and the new mutation (Koorneef et al., 1987 and 1992). Over 250 F2 seeds from each cross were sown, and resulting F2 plants analysed for the 10 phenotypic markers. However, the gl1-1 marker was unscorable in these F2 mapping populations, as the GUS negative mutations were generated in the C24 ecotype, which does not posses leaf trichomes. Therefore only 8 of the 9 markers
from W100F, L. *erecta* ecotype were scored, in these mapping experiments. To ensure a normal distribution and to validate the data for subsequent mapping calculations, each trait was evaluated for a 3:1 segregation for the entire population by \( \chi^2 \) analysis,

\[
\chi^2 \text{ formula:-}
\]

\[
\left[ \frac{(X_1 - S1)^2}{X_1} \right] + \left[ \frac{(X_3 - S3)^2}{X_3} \right] = < 3.8
\]

where, "X\(_1\)" and "X\(_3\)" are the expected no. of positive and negative individuals respectively, for a 3:1 segregation, and "S1" and "S3" are the real values.

F2 individuals, homozygous for the GUS negative mutation as indicated by male sterility, were then scored for visible markers, and again \( \chi^2 \) analysis was performed on this group of data. Linkage of the mutation to one of the markers was determined by a deviation from the expected ratio of 3:1, indicated by a \( \chi^2 \) value greater than 3.8. This value indicates a lower than expected recombination frequency between the mutation and the marker, and therefore a calculable degree of linkage. The recombination fraction, \( p \), can then be determined by the \( PR \) method, for male sterility and the associated marker, as \( PR \) relates to \( p \) and can be found in tables (Stevens, 1939), enabling the calculation of the distance between the two markers, in cM, using in this case the Kosambi mapping function.

Kosambi mapping function:-

\[
D = 25 \ln \left( \frac{100 + 2r}{100 - 2r} \right)
\]

where, "D" is the distance in cM and "r" is the recombination fraction, \( p \), expressed as a percentage.
The standard deviation for the map distance can also be calculated.

Standard deviation formula:

\[ S_D = \left[ \frac{2500}{2500 - S^2} \right] Sr \]

where, "S_D" is the standard deviation, "r" is the recombination fraction, and "Sr" is the square root of the variance, expressed as a percentage. The variance relates to the recombination fraction \( p \), and can be found in tables (Stevens, 1939).

6.6.1.1 \( gne1 \) mapping using W100F

Analysis of the data gathered from \( gne1 \) x W100F F2 individuals (Tab. 6.7), shows that no recombination occurred between the male sterility locus of \( gne1 \) and \( cer2 \). In total, over 1500 F2 plants were grown and scored for the \( cer2 \) mutation and male sterility, no recombinant individual was found. This data shows the very close linkage of male sterility gene, \( gne1 \), to the visible marker \( cer2 \), which maps to 57.0 cM on the Integrated-IV chromosome map. Therefore, the \( gne1 \) locus is located on chromosome IV, close to 57.0 cM (Fig. 6.12.a).

6.6.1.2 \( gne5 \) mapping using W100F

Mapping data from \( gne5 \) /W100F F2 individuals (Tab. 6.8) was evaluated for the segregation of the 8 markers and male sterility, using chi\(^2\) analysis (Tab. 6.9). Seven of the eight markers showed a chi\(^2\) value of less than 3.8, except \( bp-1 \) which gave a value of 6.5 and was therefore not used to evaluate a map position for \( gne5 \) in the subsequent analysis of the ms population. Chi\(^2\) analysis on the 54 ms \( gne5 \)/W100F F2 individuals generated (Tab. 6.10), showed a degree of linkage between male sterility and the \( tt3-1 \) marker, indicated by a lower than expected number of ms/\( tt3-1 \) F2 individuals, demonstrated by the deviation of the Chi\(^2\) value from 3.8 to 4.76. From the 167 F2 plants with data for ms and \( tt3-1 \), the non-parental phenotypes \( tt3-1 (+ve)/ms(+ve) \) and \( tt3-1 (-ve)/ms(-ve) \) numbered 6 and 77, and the parental phenotypes \( tt3-1 (+ve)/ms(-ve) \) and \( tt3-1 (-ve)/ms(+ve) \) numbered 39 and 45, respectively. Calculation of the product ratio, \( (6 \times 77) / (39 \times 45) \), gives a value for the argument of 0.263. Conversion of this value to the recombination fraction (Stevens, 1939), gives a value for \( p \) (in the repulsion phase) of 0.31801 or 31.8%, and a variance of 0.7832 /167, which equals an Sr value of
0.0685 or 6.85 %. Using the Kosambi mapping formula and calculating the standard deviation, the *gne5* mutation maps at a distance of 37.6 cM from the *tt3-1* marker on chromosome V, with a calculated standard deviation of 11.5 cM (Fig. 6.12.d). The *tt3-1* locus maps to 61.5 cM on the Integrated-V chromosome map. This mapping data is applicable to *gne2* and *gne3*, which are allelic to *gne5*, and the location of this mutagenised gene on chromosome V is in rough agreement with CAPS mapping data achieved from *gne2* and *gne3* (6.6.2).

6.6.1.3 *gne4* and *gne7* mapping using W100F

Similar strategies for mapping using W100F were carried out with *gne4* and *gne7*; however in the four different F2 mapping populations scored for these two mutations, the chi² values deviated from an acceptable 3:1 segregation of the markers, and no linkage was ascertained. The *gne4* mutation remains unmapped; however, the *gne7* mutation was successfully mapped using CAPS (6.6.2).

6.6.2 Mapping using CAPS analysis

CAPS (co-dominant cleaved amplified polymorphic sequences) mapping (Konieczny and Ausubel, 1993), utilises sets of PCR primers, designed for *A. thaliana*, each of which amplifies a single mapped DNA sequence. Restriction enzyme digestion of the generated DNA fragment with a determined endonuclease produces an ecotype-specific digestion pattern. CAPS was carried out on GUS negative, ms individuals, from the F2 population generated by crossing to W100F. Analysis of the resulting RFLPs allows the determination of recombination between the amplified sequence and the mutagenised locus, responsible for inactivation of the GUS gene and male sterility. Subsequently, calculation of the recombination fraction, p, from the number of recombinants within the total number of plants analysed, allows the calculation of the distance of the mutagenised locus from the mapped marker. As the GUS negative mutants were generated by irradiation of transgenic C24 *A. thaliana* seeds, the F2 individuals analysed here by CAPS, constitute a C24 and *L. erecta* background. Originally, restriction enzyme polymorphisms (Konieczny and Ausubel, 1993), were determined for Columbia and *L. erecta* ecotypes, although subsequently polymorphisms for some of the CAPS in C24 have been established. Utilising the ASA1 CAPS primers, map positions were ascertained for *gne2*, *gne3* and *gne7*. *gne7* was additionally mapped using the *PHYC* primers.
The ASA1 primers, \textit{5'}CTTACTCCTGTTCTTGCTTAC\textit{3'}, and \textit{5'}CCTCTAGCCTGAATAACAGAAC\textit{3'}, produce a 1728 bp PCR product, which on digestion with Bcl I yields an ecotype-specific polymorphism. Digestion of the ASA1 sequence from \textit{L. erecta}, produces three bands 686, 553 and 489 bp, while digestion of the C24 sequence (as for the Columbia ecotype), gives only two bands, 1042 and 686 bp in length (Konieczny and Ausubel, 1993; Altmann, T., unpublished [C24]). The PHYC primers, \textit{5'}CCTAATGGAGAATCATTCGG\textit{3'} and \textit{5'}CTACAGAATCGTCCTCAACG\textit{3'}, produce a PCR product approximately 2 kb in length, and digestion with Pst I, produces two bands of 0.8 and 0.7 kb, and several fragments of less than 0.3 kb, in \textit{L. erecta}, and 1 band of 1.7 kb, and pieces less than 0.3 kb in C24 (as for the Columbia ecotype, Poole, D., unpublished). On the Integrated-V chromosome map, the ASA1 locus maps to 14.8 cM and the PHYC locus maps to 48.9 cM. In order to estimate a map distance, of the GUS negative mutation from the CAPS marker, the recombination fraction, \( p \), can be calculated from the number of ecotype-specific alleles present, whereby:

\[
p = \frac{\text{no. of recombinant [L. erecta] alleles}}{\text{no. of total [C24 + L. erecta] alleles}}
\]

The standard deviation of the recombination fraction, \( Sp \), can be calculated, whereby:

\[
Sp = \sqrt{\frac{p (1 - p)}{n}}
\]

where \( p \) equals the recombination fraction and \( n \) equals the total number analysed.

The recombination fraction, \( p \) expressed as a percentage, can then be substituted in the Kosambi mapping function to obtain a distance expressed in cM (6.6.1).

6.6.2.1 \textit{gne2}, \textit{gne3} and \textit{gne7} mapping using the ASA1 CAPS marker

Genomic DNA was extracted from the leaves of 56 ms individuals, from the F2 \textit{gne2/ W100F} mapping population (2.9.1.2). PCR reactions (2.9.2) using ASA1 primers (2.4.3) on DNA samples were digested with Bcl I. Samples Visualised on a 1.5 % agarose gel showed that 54 out of the 56 F2 plants gave rise to only 2 bands, 1042 and 686 bp in length, indicating a homozygous
C24 restriction pattern. While the remaining 2 F2 individuals, produced four bands, 1042, 686, 553 and 489 bp in length, indicating a C24/L. *erecta* heterozygous restriction pattern. None of the 56 F2 plants analysed were homozygous for the *L. erecta* polymorphism. Therefore, from 56 *gne2*/W100F F2 ms individuals, 110 C24 alleles and 2 *L. erecta* alleles are detected, giving a recombination fraction, r, of (2/112)100, which equals 1.8 % +/- 1.3. Using the Kosambi mapping function, *gne2* maps to a distance of 1.8 cM +/- 1.3 cM from the ASA1 marker on chromosome V (Fig. 6.12.b).

From the *gne3*/W100F population, 35 ms F2 individuals were analysed by CAPS mapping using the ASA1 marker (Fig. 6.13). 24 individuals gave a C24 homozygous restriction pattern; 5 individuals gave a *L. erecta* homozygous restriction pattern and 6 plants gave a C24/L. *erecta* heterozygous restriction pattern. Therefore from the 70 alleles, 54 were derived from C24 and 16 from *L. erecta*, giving a recombination value of (16/70)100, equating to a value for r, of 22.9 % +/- 5.02. Therefore using the Kosambi mapping function, *gne3* /ASA1 CAPS data indicates a map distance of 24.7 +/- 14.2 cM from the ASA1 marker (Fig. 6.12.c).

From the *gne7*/W100F population, 43 ms F2 individuals, were analysed by CAPS mapping using the ASA1 marker, as described above (6.6.2). 24 plants were homozygous for the C24 allele, 4 plants were homozygous for the *L. erecta* allele and 15 plants were heterozygous, having both the C24 and *L. erecta* alleles. Therefore in 86 alleles, 63 were derived from C24 and 23 from *L. erecta*. These values give a recombination fraction of, (23/86)100, which equals 26.7 % +/- 4.9. Calculation of the map distance from the Kosambi mapping function, gives a distance of *gne7* from the ASA1 marker, equal to 29.8 cM +/- 6.9 cM (Fig. 6.12.f).

6.6.2.2 *gne7* mapping using the PHYC CAPS marker

In addition to ASA1 mapping (6.6.2.3), the genomic DNA extracted from the 43 ms, *gne7*/W100F F2 plants, was used to map the *gne7* mutation using the PHYC primer set. PCR reactions were carried out as described above (6.6.2). However electrophoresis of the PCR reactions from the *gne7*/W100F plants showed the absence of a 2 kb DNA fragment, or indeed any other size fragment. Homozygous C24 and *L. erecta* parental lines, analysed in conjunction, showed the expected 2 kb fragment, which on subsequent digestion with Pst I, gave the predicted polymorphic restriction patterns (6.6.2). The PCR reactions on these F2 lines and parental C24 and *L. erecta* plants, using the PHYC primers were
repeated to rule out experimental error, and indeed the same result was achieved.
Therefore in the *gne7* mutant, γ-irradiation had apparently caused a deletion of the
part of chromosome V that contains the PHYC locus. Therefore, the mutagenised
genes responsible for the *gne7* phenotype map close to the PHYC locus (Fig.
6.12.e).

6.7 *In situ* hybridisation

In order to ascertain if the cells in the GUS negative mutants, which develop
in the position normally occupied by tapetal cells in WT plants, retain their tapetal
identity *in situ* hybridisation (2.12) was carried out on anther transverse sections
from *gne1, gne2, gne4* and WT, using sense and antisense RNA probes made
from two tapetal-specific genes, *A8* and *A9*. These two probes, expected to
hybridise to the tapetal cells present in the WT anther sections, should provide a
marker(s) for the position and identity of tapetal cells in the GUS negative mutants
(particularly relevant in the case of the *gne2* mutant class), and indicate if *A8* or *A9*
are expressed in these cells. Wax embedded anther transverse sections (2.12.1)
from each mutant and WT were hybridised (2.12.4) individually, with both antisense
probes, to detect the presence of either the *A8* or *A9* transcript, and with both
sense probes to act as controls to monitor unspecific binding. Examination of the
resulting WT slides, probed with antisense constructs failed to reveal the expected
hybridisation pattern. Instead only even background staining of silver grains was
observed. Consequently, the probes failed to indicate the state of the tapetum in the
GUS negative mutants analysed.

6.8 Strategies for isolation of mutagenised genes

The *gne1* mutation maps to chromosome IV, at 57.0 cM on the integrated *A.
thaliana* map (Hauge et al., 1993), very close to the *cer2* locus. A proposed
strategy for the isolation of the *gne1* gene, is to use a targeted tagging approach,
whereby the local transposition events of a nearby maize transposable element
system is used to cause selected insertional mutagenesis (Pereira et al., S36
S.E.B. abstract 1996). A suitable homozygous *A. thaliana* line, containing an
I/dSpm transposon on chromosome IV in the vicinity of the *CER2* gene, has been
obtained from A. Pereira, which has been crossed to a homozygous *gne1* plant.
The strategy now is to screen F1 progeny for revertant ms sectors, indicating the
transposition of the mobile I element into the *gne1* gene, which can then be used
as a genetic tag to isolate the DNA bordering the transposon (Fig. 6.14). Specific primers designed against the terminal regions of the transposon are then used in IPCR to isolate fragments of the gne1 DNA, for use as a probe to screen WT genomic libraries. This will facilitate the isolation of the entire gne1 gene.

For gne7, CAPS analysis has demonstrated the presence of a deletion in this mutant, which spans the PHYC gene. The strategy for isolation of the deleted genes is to use a fragment of the PHYC gene to screen a Ti-plasmid-convertable λ phage library (Fuse et al., 1995), and isolate the PHYC containing clone, which can then be used generate a binary vector suitable for complementation analysis of the gne7 line. If complementation of the gne7 is complete, then digestion and complementation of gne7 with individual fragments of the λ PHYC clone, should enable the dissection and cloning of structural genes affected in this mutant.

The gne2 locus has been estimated to be in the region of the ASA1 marker, located on chromosome V. The fine mapping of gne2 should be achieved relatively easily, using segregation data of RFLP markers from around this region. gne4 mapping utilising the W100F marker line was inconclusive, therefore the strategy for obtaining a map position for gne4 should proceed utilising some of the numerous RFLP markers, where polymorphisms between C24 and L. erecta are available. Once an accurate map position has been obtained, map based cloning strategies can be assessed. Genomic libraries of A. thaliana are available in numerous vectors, such as yeast artificial chromosome vectors (Ecker et al., 1990; Grill and Sommerville, 1991; Ward and Jen, 1991), bacteriophage vectors (Pierce et al., 1992; Sterberg, 1990; Liu et al., 1995) and cosmids. The availability of these libraries may facilitate the cloning of GNE2 and GNE4 by chromosome walking (Putterill et al., 1993). Alternatively the discovery of a deleted marker in either mutant, could prove suitable for the direct screening of a genomic library clone, as for gne7. Another possibility, for the case of a deletion, could be the use of genomic subtraction (Strauss and Ausubel, 1990), to clone the WT deleted genes.

6.9 Discussion

In order to isolate mutants of A. thaliana affected in stamen development, we adopted a transgenic screening approach. Two homozygous, monogenic A. thaliana lines carrying different tapetal-specific promoter-GUS fusions, either A6-GUS or A9-GUS, were exposed to Cs\(^{137}\), a source of ionising gamma irradiation. Other groups have reported similar transgenic screens, using the HSP18.2
(Takahashi et al., 1992) or the CHS promoter (Jackson et al., 1995) fused to the GUS gene. In all cases, including our own, the screening of mutants for altered patterns in GUS activity, was carried out in microtitre plate wells in the presence of the GUS substrate, 4-MUG, and GUS activity was visualised as fluorescence in the presence of ultraviolet light.

The extent of mutagenesis caused by ionising radiation is known to be dependent on the dose, dose rate and oxygen and moisture content of the target tissue (Underbrink et al., 1970). Previously, Mulligan and coworkers, used a Cs\textsuperscript{137} source to generate A. thaliana mutants in male gametophyte development (Mulligan et al., 1994), subjecting seeds to a dose of 130 Krads. During trial experiments we found that such an exposure led to an unacceptable level of lethality, in the M1 population. We therefore resorted to a lower dose of 30 Krads. In the resulting M2 population, of 2 866 A. thaliana families, 1.5 % showed a detectable recessive mutation, of which just over 1 % were ms. In comparison, Mulligan and coworkers detected 10 % of the M2 population which segregated phenotypically obvious mutations, while only 0.8 % exhibited male sterility.

In screening the tapetal-specific promoter-GUS M2 population for the down regulation of the GUS transgene, 25 375 individual GUS assays were performed, and from the A6-GUS M2 population two mutant lines, gne3 and gne4 were identified, both of which lacked GUS activity in the flower buds and were ms. Screening the A9-GUS M2 population revealed 6 mutant GUS negative lines, namely gne1, gne2, gne5, gne6, gne7 and gne8. Four of the lines, gne1, gne2, gne5 and gne7 were ms, while gne6 and gne8 were male-fertile. All homozygous A6-GUS mutant lines showed a complete loss of GUS activity, except gne7, which showed 1 to 2 % GUS activity, as compared to buds from unmutated A9-GUS transgenic plants. Additionally, gne7 showed abnormal vegetative and floral development. Homozygous gne7 individuals were reduced in stature compared to WT plants, and floral morphology demonstrated abnormal numbers of petals, stamens and carpels. The morphology of the stamens in homozygous gne7 plants was also abnormal, and the anthers comprised three aberrantly shaped lobes, instead of two symmetrical theca seen in WT and the remaining GUS negative mutants. Examination of anther squashes of homozygous gne7 anthers revealed that although gne7 is ms, one or two pollen grains were found in mature anthers. In the remaining ms mutants, mature anthers were devoid of pollen grains.

All GUS negative mutant lines were outcrossed to the multimarker tester line.
W100F for mapping analysis, with pollen from the W100F line being transferred to
the stigma of each line. All GUS negative lines were found to be female fertile, and
normal silique formation and seed set took place. F1 progeny form each cross were
grown, and floral buds from each F1 line were analysed for GUS activity, to test the
integrity of the promoter-GUS transgene. In all ms GUS negative lines GUS activity
was restored, however in both the male fertile lines, gne6 and gne8, no GUS
activity was detected. These results indicated that the lesion caused by the γ-
irradiation, in gne6 and gne8, had occurred in the transgene, either within the
promoter or the GUS gene. No further analysis was carried out on these lines.

Although disappointing, these results indicate differences in the structural nature of
DNA along the chromosomes. Suggesting that some chromosomal regions may
possess a more “open” structure, preferentially allowing the integration of foreign
DNA sequences, by T-DNA mediated mechanisms. By the same token these “open”
regions may be more susceptible to attack by incoming ionising particles,
generated by irradiation. These observations are corroborated, by the fact that
different mutagens, including ionising radiation, have been found to generate
different mutant spectra, implicated by the different mutation frequencies observed
in eceriferum, gibberellin-sensitive or hypocotyl mutants (Lundqvist and Von
Wettstein, 1962; Lundqvist et al., 1962; Koornneef et al., 1982; Koornneef et al.,
1989). In addition certain loci, such as the cer-i locus, have demonstrated that they
are more susceptible, or even exclusively induced by ionising radiation (Lundqvist
and Von Wettstein, 1962).

In addition to W100F and CAPS analysis, to locate the approximate map
positions for the remaining mutants on the A. thaliana genetic map, detailed
analysis of gametogenesis was undertaken for each GUS negative mutant.
Sequential developmental series of anther transverse sections were examined
from each line, using light microscopy, to determine the timing and nature of the
lesion that results in male sterility. Examination of microsporogenesis, in these
mutant lines, showed that five of the six mutants, fell into two phenotypic classes
that showed similar if not identical lesions. One class designated gne1 -like,
comprised gne1 and gne4; while the second class designated gne2 -like
comprised gne2, gne3 and gne5. Subsequently, allelism test crosses were carried
out between the two groups. Heterozygous mutant lines for one mutation were
crossed to homozygous mutant lines in the same phenotypic class. Analysis of the
F1 progeny, showed the occurrence ms individuals in all the crossings between
In the F1 progeny produced by a cross between *gne1* and *gne4*, all individuals were male fertile, demonstrating that these two mutants are non-allelic, and contain mutations in different genes. The high frequency of mutation seen at the *gne2*-class locus, again reasserts that some genetic loci are more susceptible to attack by ionising radiation than others.

In both the *gne1*-like and *gne2*-like classes, premeiotic developmental lesions manifested in the tapetal and middle layer cells, resulted in microspore arrest and decay during meiosis, just prior to the normal development of the tetrads. In *gne7*, a third abnormal and asynchronous pattern of microsporogenesis was observed, eventually resulting in the decay of early microspores. To establish the timing of the lesions in the GUS negative mutants, with respect to three other existing sporogenous mutants, *ms32* and *ms37* and *ms33*, the A6 or A9 tapetal-specific promoter-GUS transgenes were introduced by crossing into these ms mutants. ms F2 progeny that demonstrated the presence of the transgene by PCR analysis, were assayed for the activity of the GUS gene. In all progeny tested GUS activity was apparent, indicating that the GUS negative mutants isolated carry lesions, disrupting microsporogenesis, that act earlier than *ms32*, *ms37* and *ms33*. These results imply that use of the A6 and A9 promoters provide a mechanism to look for novel mutants along the stamen differentiation pathway, which has additionally led to the identification of some of the earliest existing stamen development mutants.

Other premeiotic and meiotic mutants have been isolated and characterised (1.4.2.1), into which the tapetal-specific promoter-GUS transgenes have not been introduced. These mutants include *ms3*, *ms4*, *ms5* and *ms15* (Chaudhury et al., 1994); 7593, 7219 and 6492 (Peirson et al., 1996); *mei-1* (He et al., 1996); and *ms31* (Dawson et al., 1993, Mulligan et al., 1994) (1.4.2.1). The earliest acting mutant identified by Chaudhury and coworkers is *ms3*, where the sporogenous cells degrade at an early stage prior to a bud length of 0.5 mm. The lesion in this mutant, like the *gne1*-class and the *gne2*-class, also shows vacuolation of the tapetal and middle layer cells. Of the four mutants identified by Peirson and He and their coworkers, 7219 shows the earliest lesion, and like *gne7* this mutant shows an asynchronous patterns of development, the developmental lesion in this mutant is first apparent during meiosis. The remaining ms mutant from Dawson and Mulligan’s group, *ms31 (msK)*, shows normal meiosis, but abnormalities are seen
in callose distribution and delayed callose degradation. Therefore of all the premeiotic and meiotic mutants isolated, the GUS negative mutants, appear to cause some of the earliest lesions reported in microsporogenesis.

As a prelude to determining the nature of the lesions in the GUS negative mutants, an analysis of microgametogenesis in WT *A. thaliana* C24 was undertaken. In WT anthers, microsporogenesis follows tightly regulated programme of histodifferentiation, that can be observed as a sequence of cytological changes occurring within the sporogenous cells. These histological changes during sporogenesis, exhibit a synchronous programme of events within the four microsporangia of an anther, and between anthers of the same bud, and in addition the developmental stage of the microsporangia, shows a direct relationship to the bud length, as demonstrated for other plant species such as *N. tabacum* and *B. napus* (Koltunow et al., 1990; Scott et al., 1991b).

In *A. thaliana*, during the early stages of WT microgametogenesis, at around a bud length of 0.35 mm, the differentiated anther possesses several specialised cell and tissue types. Each microsporangium is composed of the four differentiated cell types of the anther wall, which form concentric, continuous single cell layers around the central sporogenous cells. The anther wall is composed of an external epidermis, the endothecium, the middle layer, and the tapetal cells innermost, which lie adjacent to the diploid meiocytes tightly packed in the anther locule. At around a bud length of 0.5 mm, the meiocytes enter meiosis to form callose enclosed tetrads of four haploid microspores, which are present in the locule at an approximate bud length of 0.6 mm. By this stage the middle layer cells have been flattened and degenerated. In buds around 0.7 mm in length, callose degradation has released the microspores from the tetrad, and the tapetal cells have become separate, increasingly vacuolate and started to degenerate. After this stage the microspores continue maturation, with the synthesis and deposition of the pollen wall components, and two successive mitotic divisions to form mature trinucleate pollen grains.

In WT *A. thaliana*, the development of the microsporangia is envisaged to follow a similar pattern of successive cell division from the original germ layers of the anther primordium, to that inferred through cell lineage studies carried out in *N. tabacum* and other similar plant species (Satina et al., 1940; Satina and Blakeslee, 1941; Esau, 1977; Koltunow et al., 1990). However, the genetic mechanisms involved in the establishment and regulation of pattern formation and subsequent
tissue differentiation in the microsporangia are unknown. In the three classes of GUS negative mutants identified, gne1-like, gne2-like and gne7, different aspects of microsporogenesis are affected, that may demonstrate lesions in the different types of regulation that must occur in the normal patterning and specification of the microsporangia.

In the gne1 mutant class, comprising gne1 and gne4, the initial development and histodifferentiation of the specific anther cell types is normal. In a 0.35 mm long bud from the gne1 class, the morphology of the microsporangium contains typical meiocytes, surrounded by the four differentiated cell layers of the anther wall. However, older anthers from a bud length of 0.36 mm onwards, display an abnormal and continued increase in the vacuolation of initially the tapetal and later the middle layer cells. Prior to meiosis, the middle layer cells and especially the tapetal cells have enlarged, in both size and vacuolation, and the meiocytes remain tightly packed in the anther locule, without undergoing a separation of their cell walls from the cytoplasm, normally observed in WT development. The absence of premeiosis II, in the gne1 mutant class, may already serve to illustrate the competition for space experienced by the meiocytes, due to the combined, abnormal vacuolation of the tapetal and middle layer cells.

The onset of meiosis is initiated in this mutant class, indicated by the presence of callose seen in the locular spaces between meiocytes. However, callose deposition does not result in the complete coat around each meiocyte, as in WT development. One explanation may be that space in the anther locule is now at a premium, due to the continued enlargement and vacuolation of the tapetal and middle layer cells. At this stage in the gne1 class, with bud lengths around 0.55 mm, these two innermost anther wall layers are made up of cells that possess little cytoplasm, with the majority of the cellular space being occupied by a single large vacuole. The continued gross enlargement of the vacuoles in these two cell types, seen in bud lengths around 0.75 mm, eventually leads to the abortion of the meiotic products still present in the anther locules at this stage. Ultimately in this mutant class, the distorted tetrads, tapetal cells and the middle layer of the microsporangium disintegrate, leaving empty locules. The mutation(s) responsible for the lesion seen in the gne1 class, indicate(s) that the lack of WT gene product(s), disrupts the normal differentiation of the tapetal and middle layer cells.

The gene(s), mutated in the gne1 mutant class, may normally be expressed in the tapetal and middle cell layers prior to meiosis, and may have a function in cell...
differentiation or be required for a metabolic pathway important to both these cell
types. Alternatively, if the cell lineage of the tapetal and middle layer cells originate
from a common precursor, the inner secondary parietal cells, the \textit{gne1} class of
mutation could affect the differentiation of this progenitor cell type, and
consequently, indirectly affect the normal differentiation or metabolism of the tapetal
and middle layer cells. For the latter hypothesis to have any bearing, the
microsporangium fate map of \textit{A. thaliana}, would have to resemble the cell lineages
seen in typical monocot plant species, such as \textit{Triticale} (Fig. 6.15; Bhandhari and
Khosla, 1982; Bhandhari, 1984), rather than the normal dicot pattern demonstrated
by \textit{N. tabacum} (Koltunow et al., 1990) and \textit{Anemone} (Bhandari, 1968). In normal
dicot development (Fig. 6.15), cell lineage studies indicate that the tapetal cells are
derived from the inner secondary parietal cells, and the middle layer is derived from
the outer secondary parietal cells, if this pattern holds true for the dicot \textit{A. thaliana}
species, then the former hypothesis for the WT \textit{GNE1} gene product may prove
correct.

However, the cellular phenotypes seen in the \textit{gne2} class add some weight
to the theory that the tapetal and middle layer cells in \textit{A. thaliana} are derived from a
common precursor, the inner secondary parietal cells, as in generalised monocot
development. Therefore, the latter hypothesis may be the more likely of the two
since it can explain the mode of action of the WT \textit{gne1} class of mutations. It has
been demonstrated that \textit{gne1} and \textit{gne4} are non-allelic, so although these two
mutations cause similar phenotypes, they are not a consequence of a lesion in the
same gene. Therefore the WT gene products from \textit{gne1} and \textit{gne4}, may act at a
similar time point in development of the microsporangium, or even be components
of the same pathway.

Analysis of the temporal expression patterns of \textit{GNE1} and \textit{GNE4} would
resolve if the \textit{gne1} class of mutation directly disrupts the differentiation of the
tapetal and middle layer cells or acts indirectly via their precursor cell type. Such
analysis requires the isolation of the WT genes for use in northern analysis. A
proposed strategy for the isolation of the \textit{gne1} gene, which maps very close to the
\textit{CER2} locus, is to cross a ms \textit{gne1} with a second \textit{A. thaliana} line, which contains a
nearby transposable element. F1 progeny could then be screened for revertant ms
sectors, indicating the transposable element had inserted into the \textit{gne1} locus.
Inverse PCR, could then be used to obtain a sequence of \textit{gne1} DNA bordering the
transposon, which could be used to screen a WT \textit{A. thaliana} genomic library in
order to obtain the full \textit{gne1} clone. To facilitate the cloning of the \textit{gne4} gene, this mutant must first be subject to mapping experiments, to locate this gene onto the \textit{A. thaliana} map.

In the \textit{gne2} mutant class, comprising \textit{gne2}, \textit{gne3} and \textit{gne5}, the normal development of the microsporangium is disrupted from the earliest detectable stage in anther transverse sections. To aid in the discussion of the altered development in this mutant class, the following notation has been adopted to describe the position, but not the identity, of the wall layers within the mature microsporangium (Fig. 6.16). The position denoted as S1 is normally occupied by the sporocytes, S2 the tapetum, S3 the middle layer and S4 the endothecium (Fig. 6.16). Additionally, S2/3 denotes the area normally occupied by the inner secondary parietal layer, the precursor of the tapetum and middle layer cells (Fig. 6.16). Once these cells, referred to by position show a recognisable identity such as a tapetal, middle or sporocyte cell, they are then referred to in the text by that identity. These cell identities are recognised by comparisons of mutant cell types to those found in WT anther transverse sections. Comparisons are made on the basis of cell shape and size, as well as the staining intensity of the cytoplasm and the size and position of the nucleus of the cell.

In the anthers from the smallest bud length examined (0.35 mm), from a homozygous \textit{gne2} individual, the histology of the microsporangium is already aberrant. In these anthers, the sporangial wall consists of only two or three cell layers, with only the two outermost layers, the presumptive epidermis and S4 cells, forming continuous layers. Additionally, the number of S1 cells in the anther locule is abnormally high, compared to WT anthers. As development proceeds, the disruption of cell pattern in the \textit{gne2} mutant class becomes more conspicuous. In anthers taken from buds at around 0.5 mm in length, the reduction in the numbers of cells in area S2 and S3 is apparent. Moreover, these two innermost sporangial cell types do not form continuous layers around the sporogenous cells as is found in WT. Instead, isolated islands of tapetal and middle layer cells, or a single layer of cells which might represent undifferentiated S2/3 (ISP) or middle layer is formed (S2/3).

A comparison of the numbers of each individual cell type present in a premeiotic anther transverse section from WT (Fig. 6.5.b) and \textit{gne2} (Fig. 6.7.f) was undertaken (Tab. 6.11). In the WT sporangium cross section there are 14 tapetal cells and 15 middle layer cells, indicating these cells arose from the (possibly
formative) division of 15 inner secondary parietal cells. Additionally, 5 sporogenous cells are present in the WT anther transverse section. In the gne2 anther transverse section, 10 sporogenous cells and only 2 tapetal-like and 8 of the middle layer-like cells are present. One possible explanation to account for this observation is that the division of the inner secondary parietal cells did not occur in gne2, and that 5 of these cells adopted a sporogenous cell fate, while 2 adopted a tapetal and 8 a middle layer cell fate (Fig. 6.17.a). This hypothesis would implicate a random distribution of tapetal and middle layer cells in S2/3. However tapetal cells are only observed in S2 in association with middle layer cells in S3. Therefore, an alternative hypothesis whereby only some of the inner secondary parietal cells have divided is favoured; as this observation accounts for the appearance of the isolated islands of tapetal and middle layer cells observed in gne2 (Fig. 6.17.b).

Therefore, in specific regions of the microsporangial wall in gne2 the normal configuration of four cell layers is observed (Fig. 6.7.b). In other areas, the S2 and S3 cells have not developed and the cells in their place (S2/3) have the characteristics of a sporogenous cell. Consequently, sporogenous cells may lie adjacent to endothecium cells (Fig. 6.7.c), a situation that does not arise in WT plants. This may be explained by a change in cell fate of the inner secondary parietal cells which have adopted a cellular fate more appropriate to the sporogenous cells (Fig. 6.18.a). Alternatively, the sporogenous cells may have undergone extra cell divisions resulting in these cells types juxtaposed to the endothecium cells (Fig. 6.18.b). However, the latter proposal would require an incomplete inner secondary parietal layer to allow these two cell types to come into contact. As a continuous endothecium layer was present in the gne2 anther transverse sections, formed by the outer daughter cells of the anticlinal division of the primary parietal layer, it is likely that the inner daughter cells which give rise to the inner secondary parietal layer also formed a complete layer around the sporogenous cells (Fig. 6.18.c). Thus, the former proposal whereby some inner secondary parietal cells adopt a sporogenous cell fate is more probable (Fig. 6.18.a).

In other areas of the microsporangium of gne2 a single cell layer is present between the sporogenous cells and the endothecium cells (Fig. 6.7.g), which may be either middle layer or inner secondary parietal cells. These cells are long and thin which are characteristics of middle layer cells; however without the confirmation of cell specific markers their true identity remains speculative. Despite
the varied cell patterns noted, the absence of the middle layer cells resulting in endothecium cells next to tapetal cells was not observed. This observation would suggest that the single cell layer visualised between S1 and S4 is indeed inner secondary parietal cells and not middle layer cells.

In gne2 at a bud length of around 0.6 mm, callose is present in the anther locules. The appearance of callose suggests the S1 cells have adopted a sporocyte identity and are in the initial stages of meiosis. Later in development in the gne2 mutant class, by bud lengths around 0.7 mm, the isolated islands of tapetal and middle layer cells have grossly enlarged and contain a large vacuole that occupies the majority of the cell. At this stage, the cytoplasm of the sporocytes has started to degenerate and has withdrawn from the plasma membrane. Some of the sporocytes also deteriorate prior to the completion of meiosis (Fig. 6.9.b and Fig. 6.9.c). Other sporocytes complete meiosis and degenerate prior to cytokinesis, indicated by the presence of withered meiocytes which possess cross walls and individual nuclei (Fig. 6.9.a and Fig. 6.9.c). By a bud length of approximately 0.85 mm, the tapetal and middle layer cells as well as the products of meiosis have degenerated, leaving an empty anther locule.

The observations of microsporogenesis in gne2 implicate that the WT GNE2 gene product may play a role in the assignment or interpretation of the tapetal and middle layer cell fate. Alternatively, the GNE2 gene product may be required in the formation of a boundary around the sporogenous cells, in which case a mutation in the GNE2 gene would result in the loss of this boundary, and the subsequent disruption of pattern in the microsporangium. Such postulations on the gne2 mutation do not establish the temporal nature of the lesion. However, the lesion in gne2 must have occurred prior to the differentiation of the tapetal cells, since most of these cell types are absent in the mature microsporangium. Therefore, the lesion in gne2 mutant class most likely occurred one step further back in the cell lineage, in the inner secondary parietal cells. In an attempt to justify and elaborate on these two hypotheses proposed for the mode of action of GNE2 in the developing microsporangium, comparisons of how the specification of pattern and differentiation is achieved in other plant cell types and other organisms is discussed below.

How patterning information is established and regulated in plants is relatively poorly understood. However, a number of model systems from A. thaliana, such as trichome formation and root and floral development, have been utilised to
address some of the mechanisms involved in cell type determination and
differentiation. The study of mutants affected in trichome development has identified
genes which are responsible for cell type determination and differentiation of
trichome precursor cells from epidermal cells in the shoot. Two genes \textit{GL1} and
\textit{TTG} (Koorneef et al., 1981; Koorneef et al., 1982), are required for the initiation of
trichome development, and mutations in either of these genes results in glabrous
plants. The \textit{GL1} gene has been cloned (Feldmann and Marks, 1987), and protein
analysis reveals homology to the myb class of DNA binding proteins (Oppenheimer
et al., 1991), showing greatest homology to the \textit{C1} gene in maize. The \textit{TTG} gene
has not been cloned but shows greatest homology to the maize \textit{R} gene (Lloyd et
al., 1992). This suggests that \textit{TTG} and \textit{GL1} encode cooperating myc and myb
proteins, homologous to \textit{C1} and \textit{R} of maize, that positively regulate gene
expression associated with trichome development. The clustering of trichomes
seen in \textit{ttg} mutants, ectopically expressing the maize \textit{R} gene, indicates that \textit{TTG}
may also be responsible for specifying the spatial patterning of the trichomes (Lloyd
et al., 1996), preventing adjacent precursor protodermal cells from assuming the
trichome cell fate. Additional evidence for this role of \textit{TTG}, is the formation of
clusters of trichomes in 35S-\textit{GL1} plants which have a reduced level of \textit{TTG} gene
expression (Larkin et al., 1994). Furthermore another gene, \textit{TRIPTYCHON (TRY)}
has been identified (Hulskamp et al., 1994), which is an ideal candidate for the
negative regulatory effects of \textit{TTG}, as a mutation in the \textit{TRY} gene also causes
clustering of trichome formation.

Other mutants have been identified which are required for trichome
differentiation and morphogenesis, such as \textit{GLABRA-2 (GL2)} and \textit{GLABRA-3
(GL3)} (Koorneef et al., 1982), and \textit{DISTORTED-1 (DIS1)} and \textit{DISTORTED-2
(DIS2)} (Freenstra, 1978) required for cell growth and expansion, and \textit{STICHEL
(STI), ANGUSTIFOLIA (AN), STACHEL (STA) and ZWICHEL (ZWI)} which are
required for trichome branching (Hulskamp et al., 1994).

Such mutant analysis of trichome formation, has implicated some of the
genes involved in the specification and differentiation of trichome cells, and
establishes that inductive signalling and cell-cell communication responsible for the
differentiation of cells, exists in plants, a prerequisite if either proposed action for
\textit{GNE2} is to be substantiated. However, the study of trichome mutants does not
elucidate the nature of the inductive signal responsible for the activation of such
genes, and other model systems have to be analysed to provide evidence of
signalling molecules in plant development, also required to support the GNE2 proposal.

An inductive signal required to generate the patterning of hair cells, in *A. thaliana* root epidermis, has been proposed (Cormack, 1937; Dolan et al., 1994; Dolan and Roberts, 1995; Dolan, 1996). Ethylene has long been known to act as a positive regulator in root hair development (Cormack, 1937), and the external application of ethylene causes any epidermal root cell to develop as a root hair cell (Dolan, 1996), as does the germination of seedlings in the presence of 1-amino-1-cyclopropane carboxylate (ACC), an ethylene precursor, which causes the development of ectopic root hairs in proportion to ACC concentration. In parallel, seedlings germinated in the presence of amino glycine vinyl (AVG) or silver ions which prevent the synthesis of ACC or block the cells' perception of ethylene respectively, inhibits the development of root hairs (Dolan and Roberts, 1995).

Two negative regulators of the ethylene response have been identified, *ETR1* (Chang et al., 1993) and *CONSTITUTIVE TRIPLE RESPONSE1 (CTR1)* (Kieber et al., 1991). Mutations in either gene cause ectopic hairs (Dolan et al., 1994), and both have been shown to encode protein kinases; *ETR1* encodes a protein with similarities to the two component kinases from bacteria (Chang et al., 1993), and *CTR1* shows sequence similarity to proteins from the Raf1 family (Keiber and Ecker, 1993; Keiber et al., 1993).

*ETR1*, has been proposed as a candidate for the ethylene receptor, and is a positive regulator of *CTR1*. In the *A. thaliana* root, epidermal cells that differentiate to form hair cells (trichoblasts), overlie the junction between two sub-epidermal cortical cells, while non-hair cells (atrichoblasts) overlie the outer wall of a single cortical cell. In the proposed model for trichoblast induction, local signals of ethylene are perceived through the apoplastic space, between two cortical cells causing the inactivation of *ETR1*, which therefore fails to activate *CTR1*, resulting in an activation of the ethylene signalling cascade to form a trichoblast cell. Consequently cells overlying the cortical cells do not perceive the ethylene signal, resulting in the *ETR1* and *CTR1* activation, which represses the ethylene signalling cascade resulting in a non-hair cell (Dolan and Roberts, 1995; Dolan, 1996).

Additional evidence for inductive signalling in the *A. thaliana* root, has been demonstrated through the ablation of certain cell types (Van den Berg et al., 1995). In *A. thaliana*, the primary root consists of single layers of epidermis, cortex,
endodermis and pericycle, which surround the vascular bundle. The highly organised, differentiated cells of the primary root arise by division of the four different types of initial cells, present in the root meristem, which surround the four central cells of the quiescent centre (Dolan et al., 1993). One of these groups of initial cell, the cortical initials, first divide anticlinally to form two cortical daughter cells, which then divide periclinaly in a formative division, that gives rise to cortex and endodermal cells (Dolan et al., 1993, Scheres et al., 1994).

The use of laser ablation to eradicate a cortical initial cell (Van den Berg et al., 1995), results in adjacent pericycle cells invading the vacant space. The invading pericycle cells then divide periclinaly to maintain the pericycle cell file and the cortical cell file. The formative pericycle cell in the cortical cell position, then undergoes the normal formative division expected of a cortical initial cell, to form the cortex and endodermal cells. The change in fate of the pericycle cells, in response to their new position, demonstrates developmental plasticity, and indicates inductive signalling and cell-cell interactions play an important role in plant development. This demonstrates that it is the position of the cell with respect to the inductive signal, and not the cell lineage, which is important in the specification of cell identity. To determine the direction of the inductive signal, laser ablation was applied to three cortical daughter cells (Van den Berg et al., 1995). Monitoring of the isolated cortical initial underneath revealed the lone initial cell was still able to undergo proliferative divisions to generate daughter cells, but was unable to perform asymmetric, formative divisions to generate the normal cortex and endodermis cells. From this observations, Van den Berg and coworkers, concluded that meristem initial cells are not the generators of patterning information, but rather perpetuate an existing pattern through inductive signalling from the mature daughter cells, directed towards the root meristem tip.

These experiments may implicate an inductive signal that originates from the sporogenous cells and is radially propagated outwards to the other cell types and directs the specification of pattern in the microsporangium (Fig. 6.19.a). This signal may specify a primary state, directing all the cells of the microsporangium to differentiate as sporogenous cells, and the GNE2 gene may act to repress this signal thereby allowing the expression of a tapetal and middle layer cell fate. Therefore, in a simple scenario for gne2 mutants a complete transformation of tapetal and middle layer cells types to sporogenous cells would be expected, presuming gne2 is a null mutation. However, some tapetal and middle layer cells
are formed which may implicate that the gne2 mutation is not null and a threshold level of GNE2 expression maybe required for WT development.

Evidence of inductive signalling has also been demonstrated in the floral meristem of A. majus, using a temperature sensitive Tam3 transposon inserted into the promoter of the pal gene. This gene is required for the red pigmentation of the epidermal cells of the petals, stamen filaments and carpels (Vincent et al., 1995). During different stages of development, plants grown at 25 °C were exposed for short periods to 15 °C, allowing the activation of the Tam3 transposon, and the pigmentation of clones of cells, which were then followed during subsequent development. Marked clones of cells, induced before the emergence of the sepal primordia, were not restricted later in their development by any whorl boundaries, but were able to contribute to more than one type of floral organ, indicating that initially cells are not committed with respect to floral organ identity. However, after the formation of the sepal primordia, lineage restrictions between adjacent whorls started to appear, indicated by the restriction of marked cells to individual whorls that were unable to cross the boundaries between the whorls (Vincent et al., 1995). Therefore the formation of boundaries has been implicated in the differential development of equipotential cells, which may implicate a role for the GNE2 gene.

RNA in situ hybridisation shows lineage restrictions between adjacent whorls start to appear around the time when the expression domains of the organ identity genes has become clearly defined (Wiegel and Meyerowitz, 1994). The floral homeotic organ identity genes, may be responsible for establishing cell lineage boundaries between adjacent whorls through the interaction with another set of genes. One candidate in A. majus is the FIMBRIATA (FIM) gene which is proposed to activate the organ identity genes, and then later becomes restricted to localised regions in the second whorl, three to four cells across in junctions between the adjacent whorls one and three (Simon et al., 1994).

In A. thaliana, another candidate proposed in the regulation of cell boundaries is the SUPERMAN (SUP) gene, although as opposed to the FIM gene, SUP gene activation is thought to occur via the organ identity genes (Sakai et al., 1995). sup mutants contain extra stamens in the third whorl and reduced carpelloid tissue in the fourth whorl, accompanied by the ectopic expression of AP3 and PI, closer to the central region of the flower than is found in WT plants (Schultz et al., 1991, Bowman et al., 1992). These findings led to the proposal that SUP was an upstream negative regulator of B function genes, AP3 and PI, in the fourth whorl.
However, cloning of the SUP gene, and in situ hybridisation with a SUP antisense probe in WT floral primordia, showed that SUP expression is limited to the third whorl cells, and is coincident with a subset of the AP3/PI expressing domain, at a boundary between the third whorl stamens and the fourth whorl carpels (Sakai et al., 1995). Therefore it is proposed that SUP acts either by forming a boundary between the third and fourth whorls, or SUP normally acts to repress cell division thereby preventing proliferation of AP3/PI expressing cells in the third whorl, at the boundary between the third and fourth whorls (Sakai et al., 1995). To explain the reduction of the fourth whorl carpels, seen in sup mutants, Sakai and coworkers additionally postulate that the excessive cell division in the third whorl, or some other non-autonomous function of SUP, results in the repression of cell division in the fourth whorl. Sequence analysis of the SUP protein, revealed motifs suggesting SUP acts as a transcription factor (Sakai et al., 1995), therefore the molecular nature of the inductive signal transmitted between the two whorls remains unanswered. In the gne2 mutant class, the apparent increase in the numbers of sporogenous cells at the expense of the presumptive tapetal and middle layer cells, raises the question whether GNE2 has any functional homology to the SUP cadastral gene. If so, this may implicate the GNE2 gene product, either directly or indirectly, in the regulation of cell boundaries between the sporogenous cells and the tapetal and middle layer cells.

As the establishment and regulation of pattern is poorly understood in plants, how such signalling is achieved in animals, must be considered. A great deal of knowledge on the elaboration of pattern has come from studies on the fruit fly, D. melanogaster, and the nematode, C. elegans. In the embryos of these two organisms, the asymmetrical distribution of maternal morphogens along the anteroposterior and dorsoventral axes specifies positional information to the cells (Mello et al., 1994; Lawrence and Struhl, 1996). In D. melanogaster, one of the maternal mRNAs, BICOID, is localised to the anterior pole of the egg, which becomes translated after fertilisation. The protein product then diffuses away through the initial syncytium of the embryo creating a concentration gradient along the anteroposterior axis (Berleth et al., 1988; Driever et al., 1990). The BICOID gene, encodes a transcription factor which along with the gap genes initiates the pair rule genes, FUSHI TARATZU and EVENSTRIPED, in alternating stripes along the embryo. The pair rule genes initiate the segment polarity genes (homeotic
selector genes) (Garcia-Bellido, 1975), which in turn establish further morphogen
gradients, which elaborate cell specification and determination.

The formation of the larval wing disc is specified from a group of founder
cells that straddle the border between parasegments 4 and 5. The posterior cells, of
the wing disc, express the segment polarity gene *ENGRAILED (EN)*, while the
anterior cells do not. The *EN* gene initiates *HEDGEHOG (HH)* expression which
encodes a secreted protein, with short range morphogenic effects (to which cells
expressing *EN* are insensitive). The hedgehog protein crosses over the border to
the anterior side where it activates *DECAPENTAPLEGIC (DPP)*, which encodes a
long range morphogenic protein that diffuses away anteriorly and posteriorly to
create concentration gradients, capable of inducing the typical cell patterning seen
in the wing. Evidence of similar pattern regulation is seen in vertebrate limb
formation; *SONIC-HEDGEHOG*, a homologue of *D. melanogaster HEDGEHOG*,
encodes a secreted signalling molecule that organises the anteroposterior pattern
(Riddle et al., 1993). Moreover it may induce the expression of bone morphogenic
proteins 2 (BMP2) and BMP4, homologs of DPP (reviewed by Roelink, 1996). There
is also evidence for graded morphogens in *Xenopus*, where embryonic mesoderm
induction, in the dorsoventral axis, specifies five different cell fates, determined by
the secreted protein, activin (Green and Smith, 1990). Activin, also shows
homology to DPP and bone morphogenic proteins, which together belong to the
transforming growth factor β (TGFβ) family of cytokines (Massague, 1996).

Other diffusible morphogenic factors such as retinoic acid, have been
implicated in *Xenopus* and mouse embryos (reviewed by Jessell and Melton,
1992). Homologs or secreted proteins related to these families from vertebrates,
have not been discovered in plants, however, some of these signalling molecules
may turn out to be carbohydrates rather than proteins, as illustrated by the induction
of epidermal root hairs, by ethylene in *A. thaliana* (Dolan et al., 1996). Therefore in
the *A. thaliana* microsporangium, an inductive signal could be in the form of a
concentration gradient of morphogenic potential (Fig. 6.19.b), as demonstrated in
the specification of pattern in different animal species. Alternatively, a signal
emanating from the sporogenous cells could be laterally propagated from one cell
type to the next, which may be the more likely of the two proposals considering the
differences in the nature between animal and plant cell walls (Fig. 6.19.b).

In a comparison of *D. melanogaster* and floral development, the cadastral
genes, such as *SUP* and *LUG*, from plants appear to play a similar role in the
regulation of the floral homeotic genes, as the gap genes do in the control of the homeotic genes in *D. melanogaster*. Both the gap genes and the cadastral genes are involved in the regulation of segmental boundaries (Wiegel and Meyerowitz, 1994). Mutations in the gap gene *tailless* from *D. melanogaster*, cause an expansion of the central region at the expense of the tail (Mahoney and Lengyel, 1987), just as *sup* mutations cause an expansion of the third whorl stamens at the expense of the fourth whorl carpels. In an analogy drawn from the establishment of pattern in *D. melanogaster*, one might envisage the tapetal and middle layer comprising a single compartment, whereby the *GNE2* gene product may establish a single boundary between these two cell types and the sporogenous cells, and another gene product coupled to *GNE2* expression may be required for the differentiation between the tapetal and middle layer cells, which make up two segments contained within the compartment. On the other hand, expression of *GNE2* in the tapetal cells, may establish two boundaries, one between the tapetal and sporogenous cells and the other between the tapetal and middle layer cells. In either possibility, mutation of the *GNE2* gene would result in the loss of boundaries between the sporogenous cells and both the tapetal and middle cell layers, causing most of these cell types to differentiate as sporogenous cells, analogous to the change in cell fate seen in *sup* mutants.

The isolation and cloning of the *GNE2* gene will clarify the proposed mode of action of this gene. The analysis of the temporal and spatial patterns of expression of *GNE2*, and the protein sequence which may demonstrate some motifs, will elucidate the functional nature of the protein and determine whether *gne2* has a role in either cell specification or boundary formation. Mapping data from this class of mutants, indicates that the *gne2* gene class is located on chromosome V, in the proximity of the ASA1 marker. Future fine mapping of the *gne2* locus should enable the cloning of the mutagenised gene by map based techniques.

In *gne7* mutant, microgametogenesis displays an asynchronous pattern of development. While the gamete cells within a single locule display a synchronous pattern, cells between locules of the same anther and between anthers of the same floral bud show an asynchronous developmental pattern. Simultaneous analysis of megagametogenesis, in the gynoecium, shows the asynchronous phenomenon is specific to the male gametes, as ovule development in one ovary of the fused carpel, mirrors the stage of ovule development in the second adjacent ovary, and
synchrony is maintained throughout gynoecium maturation, in the floral buds examined from heterozygous \textit{gne7} plants. Additionally in \textit{gne7}, the onset of meiosis is delayed, and occurs at later bud lengths than compared to WT development, where meiosis is synchronously initiated at around a bud length of 0.5 mm. The earliest observation of meiosis in \textit{gne7}, indicated by the presence of callose, is seen at buds lengths around 0.65 mm, while in other locules, meiosis remains uninitiated as late as a bud length of 0.76 mm. As opposed to the lesions seen in the \textit{gne1} and \textit{gne2} mutants, the histology and development of the sporangial wall remains unaffected in \textit{gne7}, and in buds around 0.5 mm in length, the stereotypical arrangement of the four anther cell types is visualised. The tapetal and middle layer cells, follow their normal progression of degeneration, according to the developmental stage of the gamete cells in the anther locule. In one anther, taken from a 0.76 mm long bud, one locule shows premeiotic meiocytes surrounded by separate but intact tapetal cells, which overlie a flattened but still present middle layer, while in an adjacent locule, tetrads are present surrounded by degenerating tapetal cells in the absent of any middle layer. In a single bud 0.95 mm in length, the final abnormal stages of gamete development in \textit{gne7}, can be seen. One locule contains tetrads, another early microspores, and a third shows degenerating microspores, where the plasma membrane enclosing the cytoplasm has shrunk away from the newly formed exine wall. Due to the asynchronous initiation of meiosis, this pattern initially observed in an 0.95 mm bud, continues to be the norm and is repeated in buds as late as 1.27 mm in length. Therefore although meiosis is initiated at different stages in the anther locules, all the gamete cells follow the same degenerate pathway, resulting in microspore abortion shortly after the deposition of the exine wall. After a bud length of 1.27 mm, examination of anther transverse sections reveals locules where the tapetal and middle layer have completely degenerated, and the occasional degenerate microspore. The asynchronous initiation of meiosis demonstrated in \textit{gne7}, implicates a genetic lesion originating in the tapetum, which has a WT function in the regulatory mechanism involved in the imposition of synchrony in the meiocytes. In support of this proposition, is the observation that the synchronous initiation of meiosis in WT development, is achieved by some factor(s), that originate from outside the sporogenous cells (Walters, 1985), and the meiocytes are only capable of developing into mature pollen grains, \textit{in vivo}, without the influence of the tapetal cells, if they have already reached the leptotene-zygotene stage of meiosis (Ito and
Stern, 1967). Furthermore, the control of meiotic synchrony is thought to be imposed by the accumulation of the sporogenous cells and slightly later the tapetal cells, at the G1 stage of meiotic interphase, from which hold both cell types are simultaneously released, entering DNA synthesis together. The factor(s) responsible for the accumulation and release of these two cell types from the G1 stage, are proposed to act via plasmodesmata connecting the tapetal cells to the meiocytes, creating a syncytium through which the regulatory molecule(s) can freely pass (Heslop-Harrison, 1966a, 1966b). Therefore one of the mutations in gne7, isolated by the down regulation of a tapetal specific gene, may disrupt one aspect of the pathway responsible for the synthesis of such a hypothesised factor, and could encode an enzyme responsible for the synthesis of precursors or the factor itself. Two previously identified ms mutants, 7593 and 7219 (Peirson et al., 1996), demonstrate asynchronous pollen development, and a failure to produce callose. Peirson and coworkers, suggest that callose may be necessary for imposition of synchrony in meiosis. The observation of callose production in gne7, demonstrates that if callose is required for the imposition of synchrony, it is not the only requirement and other regulatory factors are needed.

CAPS mapping on the 43 ms F2 gne7/W100F individuals using the ASA1 marker, locates the gne7 mutation to 29.8 cM +/- 6.9 cM from the ASA1 marker, which is located at 14.8 cM on the Integrated-V chromosome map. CAPS mapping using the PHYC mapping primers, showed gne7 to contain a deletion which spans the PHYC locus. The PHYC gene is located at 48.9 cM on the integrated-V chromosome map, which validates the mapping data obtained from the ASA1 markers, as the PHYC locus falls within the bounds of the standard error calculated for the distance of gne7 from the ASA1 marker.

To estimate the size of the deletion generated in gne7, bacteriophage DNA was prepared from a λ 5.2 clone, containing 14 kb of DNA sequence spanning the PHYC gene. The entire 14 kb of WT A. thaliana sequence contained in the λ clone, was excised using the Sal I restriction endonuclease, and labelled using radioactive 32P, for use in southern analysis on WT and gne7 genomic DNA. The autorad, exposed for 24 hrs, indicated that the entire 14 Kb fragment had been deleted in gne7. Further southern analysis, using labelled end fragments from the λ 5.2 clone, subsequently showed that at least 2.85 kb of the 5' region of the clone is present in gne7, however southern analysis on the 3' end was inconclusive, and therefore part of this end of the clone may or may not be present.
A proposed strategy for cloning the WT sequences deleted in gne7 was initiated, but remains uncompleted. A BamHI fragment of the PHYC gene was cut from the λ 5.2 clone, radiolabelled and used to probe a λTI2-plasmid-convertible phage cDNA library (Fuse et al., 1995). The isolation of the PHYC λTI2 clone was unsuccessful. However, the future isolation of this clone could enable the direct complementation of the gne7 mutant; as phage infection of the recombinant cre/lox PHYC λTI2 clone, into the cre+ E. coli strain NS5329 (Elledge et al., 1991), would result in a binary vector directly suitable for A. tumefaciens mediated transformation into A. thaliana (Sternberg and Hamilton, 1981). If complementation of gne7, by the PHYC λTI2 clone was complete, then complementation analysis of gne7 with individual fragments of the clone would enable the identification of genes responsible for each individual phenotype visualised in gne7.

Transgenic pollen from a homozygous 35S-PHYC sense A. thaliana line was used to pollinate a ms gne7 individual, and 100 F2 seeds were germinated (Devlin et al., unpublished), of which 24 progeny had a gne7 phenotype (reduced stature, ms). PCR analysis using primers designed against the PHYC or 35S promoter, were used to check for the presence of the native PHYC gene and the 35S-PHYC transgene respectively. 18 of the 24 gne7 F1 plants had the 35S-PHYC transgene present, as did the remaining plants with a WT phenotype (Devlin et al., unpublished). These data demonstrate that the 35S-PHYC transgene does not rescue the gne7 phenotype, and that other gene(s) deleted in the gne7 mutant, in close proximity of the PHYC gene are responsible for the reduced stature and male sterility phenotypes.

Therefore the future isolation and transformation of gne7 with the PHYC clone, containing adjacent DNA sequences from the λTI2 library, should enable the complementation of the gne7 phenotype. Transformation of gne7 with fragments of this clone, should then facilitate the ultimate cloning of the genes responsible for the individual phenotypes, one of which may have a specific role in the control of meiosis synchronisation in the male gametes.
6.10 Future work

To date the phenotypic and genotypic characterisation of the GUS negative mutants, has lead to the isolation of four non-allelic mutants, \textit{gne1}, \textit{gne2}, \textit{gne4} and \textit{gne7}. Observations on mutant lines have shown vegetative and floral phenotypes, and revealed mutant anther histology. Genetic analysis has elucidated the fine map positions for \textit{gne1} and \textit{gne7}, and a rough map position for \textit{gne2}, while \textit{gne4} has yet to be allocated to one of the five chromosomes. Future analysis should therefore involve the fine mapping of \textit{gne2} and \textit{gne4}, which can then be evaluated for a suitable map based cloning strategy, with a view to cloning the genes responsible for the mutant phenotypes in the four GUS negative mutants, confirmed by complementation analysis. Subsequently, long term future work must involve DNA sequencing, and analysis of promoter and coding regions, leading to the elaboration of protein sequence and structure. Homology searches on the available DNA and protein databases, combined with the temporal and spatial gene expression patterns in WT plants, may then offer clarification of gene function in these ms mutants.

6.11 Conclusions

A transgenic screening approach was utilised to identify mutants of \textit{A. thaliana} affected in stamen development. Transgenic \textit{A. thaliana} seed, homozygous for either an A9-\textit{GUS} or A6-\textit{GUS} transgene, were exposed to a Cs$^{137}$ source of $\gamma$-irradiation. The resulting M2 families were screened for the down regulation of the A9 or A6, tapetal specific promoter activity, by assaying for the loss or reduction of GUS activity in the floral buds.

Four non-allelic mutant lines, affected in stamen development were identified, designated \textit{gne1}, \textit{gne2}, \textit{gne4} and \textit{gne7}, all of which are ms. Analysis of microsporogenesis for each mutant line, revealed that \textit{gne1} and \textit{gne4} are required for the normal differentiation of the tapetal and middle layer cells, while \textit{gne2} is involved in the specification or control of identity of these two cell types. The third mutant, \textit{gne7}, shows the asynchronous development of the sporogenous cells between adjacent anther locules, therefore one of the \textit{gne7} gene products has been implicated in the synchronous control of meiosis during microgametogenesis.

The \textit{gne7} mutant was found to contain a large deletion spanning the \textit{PHYC} locus on chromosome V. The deletion of the \textit{PHYC} gene, was shown not to be
responsible for the male sterility and other associated phenotypes of \textit{gne7}. Genetic mapping located the lesion in the \textit{gne2} mutant to 15.6 cM on the Integrated-V chromosome map, while \textit{gne1} mapped close to 57.0 cM on the Integrated-IV chromosome map. The \textit{gne4} mutant still has to be allocated a map position.
Fig. 6.1 Irradiation dosage experiment.

(top figure)
A9-GUS transgenic seedlings, germinated from seed exposed to varying doses of caesium$^{137}$ radiation,

a. non-irradiated
b. 30 Krads
c. 60 Krads
d. 90 Krads
e. 120 Krads.

Fig. 6.3 Microtitre plate GUS assays.

(bottom figure)
Positive GUS activity was visualised as fluorescence under ultra-violet illumination. Each row represents a series of individuals from a single M2 family. The absence of GUS activity in wells H4 and H5, indicates the down regulation of the GUS gene, seen here in gne1.
**Fig. 6.2** Examples of phenotypically obvious mutants obtained in a M2 family.

Visual mutants identified during the screening of the A6-GUS and A9-GUS mutagenised populations.

M2 families, showing the segregation of homozygous mutants,

a. chlorophyll mutant
b. growth inhibited mutant.

Individuals showing phenocopies of previously identified *A. thaliana* mutants,

c. embryo lethal mutant
d. cactus
e. pin-formed
f. *ap1*

**g. hy1/hy2** (left), C24 WT (right)
h. *ap2*. 
Fig. 6.4 Phenotypic analysis of homozygous gne7 mutants.

(a.) gne7 individuals, show a reduced stature and an increase in branching, as compared to WT C24 plants.

The primary leaves in gne7 seedlings (c.), are smaller than C24 WT (b.) at the same developmental stage.

Scanning electron micrographs of WT C24 (d.) and gne7 (e.) flowers, demonstrate the abnormal numbers of floral organs found in gne7. The anthers of gne7 (g.) possess three misshapen lobes, instead of the two symmetrical theca found in WT counterparts (f.).
Fig. 6.5 Microsporogenesis in WT *A. thaliana*.

0.5 μm anther transverse sections from wild type *A. thaliana*, C24, stained with toluidine blue. Bud lengths are in brackets. Scale bar = 25 μm.

a. Early microsporangium (0.39 mm). Angular meiocytes (M) surrounded by the microsporangium wall - the tapetum (Ta), the middle layer (Mi), the endothecium (En) and the epidermis (E).

b. Pre-meiosis I (0.44 mm). The tapetum has increased in size and cells contain vacuoles (V).

c. Pre-meiosis II (49 mm). Separation has occurred between the cytoplasm and the microsporocyte wall (▲).

d. Tetrads (T) (0.56 mm). Meiosis forms tetrads of four haploid microspores encased within a callose coat (▲). Tapetal cells (Ta) are large, separate and vacuolate. Middle layer (Mi) is flattened and squashed.

e. Microspore release (0.69 mm). Free microspores (ms) are present in the anther locule. The tapetal cells (Ta) are degenerating. Middle layer flat (▲). Pollen wall formation (0.7 mm). Exine coat of microspores stains darkly (▲). The tapetal cells (Ta) have almost completely degenerated and stain very faintly.
Fig. 6.6 Microsporogenesis in *gne1* and *gne4* mutants.

*gne1* and *gne4* are non-allelic. However, both these lines demonstrate a similar mutant phenotype in microsporangium histology, and therefore their anther transverse sections have been grouped together. 0.5 μm anther sections from *gne1* (a-h) and *gne4* (i-l) stained with toluidine blue. Bud lengths are in brackets.

Scale bar = 25 μm.

**a.** Early microsporangium *gne1* (0.35 mm). Meiocytes (M) surrounded by the tapetum (Ta), middle layer (Mi), endothecium (En) and the epidermis (E).

**b.** *gne1* (0.36 mm) and **i.** *gne4* (0.49 mm). Angular meiocytes (M). The tapetal and middle layer cells have increased in size and some contain vacuoles (V).

**c.** *gne1* (0.46 mm) and **j.** *gne4* (0.54 mm). The vacuoles (V) present in tapetal and middle layer cells have increased in size. No pre-meiosis II.

**d.** *gne1* (0.51 mm) and **k.** *gne4* (0.6 mm). Meiocytes (M) enter meiosis and have secreted callose (Ca). The tapetal and middle layer cells have increased in size, with the majority of the cells occupied by a single, large vacuole.

**e.** Comparative longitudinal section of *gne1* (0.44 mm). Callose (Ca) fills any spare locular space and meiocytes are tightly packed together. Tapetal and middle layer cells contain large vacuoles (V).

**f.** *gne1* (0.75 mm) and **l.** *gne4* (0.6 mm). Tapetal and middle layer cells contain a grossly enlarged vacuole and very little cytoplasm. Abnormal tetrads (T), partially surrounded by callose, have become distorted.

**g.** *gne1* (0.89 mm) - tapetal cells (Ta) have further enlarged and become elongated, crushing the misshaped tetrads.

**h.** *gne1* (0.95 mm) - remnants of tapetal cells persist (▲), while the tetrads have completely disintegrated, leaving an empty anther locule.
Fig. 6.7 Microsporogenesis in gne2 mutants.

0.5 μm anther sections from gne2 stained with toluidine blue. Bud lengths are in brackets. Scale bar = 25 μm.

a. Abnormal early microsporangium (0.35 mm). Disrupted anther wall. Only presumptive epidermis (E) and endothecium (En) cells form continuous layers. Numerous meiocytes (M) pack the anther locule. b. (0.43 mm). Four cell layers of the microsporangium are visible in specific regions (►◄), creating isolated islands of tapetal and middle layer cells. c. (0.47 mm). Microsporangial wall consists of only two or three cell layers across. Where the microsporangial wall is only two cell layers deep, the meiocytes develop alongside endothecium cells (▲).

d. (0.53 mm). Isolated tapetal cells are visible (Ta).

e. (0.53 mm). Pre-meiotic meiocytes (M) still tightly pack the anther locule.

f. (0.57 mm) An island of tapetal and middle layer cells is visualised concentric with the central core of meiocytes (arrows). g. (0.55 mm) Tightly packed meiocytes secrete callose (Ca). Where the microsporangium wall is three cell layers deep, the meiocytes lie next to middle layer like cells or possibly inner secondary parietal cells (▲).

h. (0.48 mm) Some meiocytes (M) have become separate, and callose (▲) is deposited in the locular spaces. i. (0.69 mm) Post-meiotic initiation. The meiocytes (▲) are surrounded by callose. The tapetal cells are grossly enlarged and the majority of the cell is occupied by a single vacuole (V). j. (0.75 mm). The tapetal cells (Ta) have increased in size and encroach upon the locular space. The products of meiosis have become distorted. k. (0.8 mm). The tapetum and meiotic products are degenerating. l. (0.9 mm). The contents of the locule have disintegrated and only remnants of the tapetal cells remain (▲).
Fig. 6.8 Microsporogenesis in gne3 and gne5 mutants.

gne2, gne3 and gne5 are all allelic, and share the same mutant microsporangium histology. Therefore, only six anther sections from each of gne3 and gne5 have been displayed, to reinforce the phenotype visualised in gne2. 0.5 µm anther sections from gne3 (a-f) and gne5 (g-l) stained with toluidine blue. Bud lengths are in brackets. Scale bar = 25 µm.

- a. gne3 (0.49 mm) and g. gne5 (0.48 mm). Tapetal (Ta) and middle layer (Mi) cells do not form continuous layers around the meiocytes. h. gne5 (0.59 mm). Numerous meiocytes (M) pack the anther locule. b. gne3 (0.63 mm), i. gne5 (0.62 mm) and j. gne5 (0.60 mm). Isolated islands of tapetal and middle layer cells are visualised (arrows).
- c. gne3 (0.55 mm) and k. gne5 (0.57 mm). Darkly staining callose is deposited in any remaining locular space.
- d. gne3 (0.64 mm). Isolated tapetal cells (Ta) contain large vacuoles. e. gne3 (0.58 mm). Post-meiotic initiation. Degenerating meiocytes are present (▲) surrounded by callose. f. gne3 (0.58 mm) and l. gne5 (0.90 mm). Tapetal cells (T) are grossly enlarged and vacuolate. The misshapen products of meiosis are squashed and have started to degenerate.
Fig. 6.9 *gne2* and *gne5* showing the degradation of the meiotic products of microsporogenesis.

0.5 μm anther sections from *gne2* (a-b) and *gne5* (c-d) stained with toluidine blue. Bud lengths are in brackets. All sections at the same magnification. Scale bar = 10 μm.

**a. gne2** (0.61 mm) and **c. gne5** (0.73 mm). Post-meiotic, pre-cytokinesis, degenerating meiocytes are formed, indicated by the presence of cross walls (C) and individual nuclei (N). **b. gne2** (0.54 mm) and **d. gne5** (0.81 mm). The meiocytes (M), which have secreted callose (Ca), have initiated but fail to complete meiosis.
Fig. 6.10.a Microsporogenesis in gne7 mutants.

0.5 μm bud transverse sections from gne7, showing simultaneous microsporangia and gynoecium development. All sections at the same magnification. Scale bar = 25 μm.

a. and b. 0.35 mm bud length:-

a. Early anther. The microsporangium is not fully differentiated and comprises epidermal (E) and parietal cells (P).

b. Ovule development in the gynoecium has not been initiated.

c. and d. 0.5 mm bud length:-

c. Pre-meiosis. Meiocytes (M) are surrounded by the four anther cell wall layers, the tapetum (Ta), the middle layer (Mi), the endothecium (En) and the epidermis (E).

d. Ovules emerge as a dome of cells.

e. and f. 0.65 mm bud length:-

e. Meiosis. Meiocytes secrete callose (▲). Tapetal cells (Ta) are large separate vacuolate cells. Middle layer (Mi) has become flattened.

f. Ovules show synchronous extension (0.65 mm).

g. h. and i. 0.76 mm bud length:-

g. Pre-meiosis. Meiocyte surrounded by large separate tapetal cells (Ta).

h. Post-meiotic tetrads (T). Callose encased tetrads surrounded by degenerate tapetal cells.

i. Gynoecium differentiation shows tight synchronous development of the ovules.

j. k. l. and m. 0.95 mm bud length:-

j. Tetrads (T). Tapetal cells are large and vacuolate.

k. Microspore release. Early microspores (ms) present in the anther locule. The tapetal cells are degenerating.

l. Exine (▲) has been deposited around the prematurely degenerating microspores. Plasma membranes have detached from the exine walls, and microspore cytoplasm has shrunk and is degenerating.
Fig. 6.10.b Microsporogenesis in gne7 mutants - continued.

0.5 μm bud transverse sections from gne7, showing simultaneous microsporangia and gynoecium development.
All sections at the same magnification. Scale bar = 25 μm.

m. Ovule development occurs simultaneously in the gynoecium.

n. o. and p. 1.08 mm bud length:-
n. Exine is clearly visible around the intact microspores (▲). Tapetal cells (Ta) are large and intact. o. The microspores are degenerating. Plasma membranes have dissociated from the exine walls (▲). The tapetal cells have started to degenerate (Ta).
p. Ovule development shows continued development from the previous section from a 0.95 mm long bud.

q. r. and s. 1.27 mm bud:-
q. Intact microspores with an exine coat (▲). The tapetal cells are degenerating. r. Degenerating microspores. Microspore cytoplasm has withdrawn from the exine coat (▲). The tapetal cells are disintegrating (Ta). s. Ovules have simultaneously increased in length and breadth.
**Fig. 6.11** T.E.M. of anther transverse sections of C24 WT and *gne1*.

Micrographs of the anther wall from C24 WT (a.) and *gne1* (b.), showing the epidermis (E), endothecium (En), middle layer (Mi), tapetum (Ta) and meiocytes (M). 2.6 x 10^3 magnification.

Detail of a tapetal cell from C24 WT (c. 6.6 x 10^3 magnification) containing numerous small vacuoles (V). A tapetal cell from *gne1* (d. 5.0 x 10^3 magnification) contains a single large vacuole (V).

Comparison of ribosome number in tapetal cells from C24 (e.) and *gne1* (f.) at 100 x 10^3 magnification, shows the reduction of ribosomal bodies found in *gne1* homozygous mutants.
Fig. 6.12 Genetic maps of chromosomes IV and V, showing the estimated map positions for gne1, gne2 and gne7.

map position of:-

a. gne1  (W100F data)
b. gne2  (ASA1 CAPS data)
c. gne3  (ASA1 CAPS data)
d. gne5  (W100F data)
e. gne7  (PHYC CAPS data)
f. gne7  (ASA1 CAPS data).
**Fig. 6.13** CAPS analysis of *gne3*/W100F F2 *A. thaliana*.

CAPS mapping analysis of 35 male-sterile F2 individuals, from the *gne3*/W100F population using the ASA1 marker. 1.5 % agarose gel, showing Bcl II digested PCR ecotype specific polymorphisms,

\( \Delta \) = RFLP for WT C24

\( \Box \) = RFLP for WT *L. erecta*

\( \blacktriangle \) = RFLPs for homozygous C24 plants from the *gne3*/W100F mapping population

\( \blacksquare \) = RFLPs for homozygous *L. erecta* plants from the *gne3*/W100F mapping population.

\( \odot \) = RFLPs for heterozygous C24/L. *erecta* plants from the *gne3*/W100F mapping population

\( \bullet \) = 1 Kb λ DNA ladder
Fig. 6.14 Strategy for the isolation of the *gne1* gene.

A homozygous *gne1* mutant has been crossed to another *A. thaliana* line, containing an *I/dSpm* transposon on chromosome IV in the vicinity of the *CER2* gene, which maps close to the *gne1* locus. The proposed strategy is now to screen the F1 progeny for revertant ms sectors indicating the mobile *I* element has transposed into the *gne1* gene, which can then be used as a genetic tag to isolate the DNA bordering the transposon using IPCR. IPCR fragments can then be used to screen genomic libraries to isolate a clone containing the entire *gne1* gene.
male sterile transposon containing line 1 in 10,000 F1 lines
Fig. 6.15 Cell lineage of the microsporangium in typical monocot. and dicot. plant species.

Abbreviations: A, archesporial cell; PP, primary parietal cell; PS, primary sporogenous cell; OSP, outer secondary parietal cell, ISP, inner secondary parietal cell; En, endothecium; Mi, middle layer; Ta, tapetum; S, sporocytes.
Fig. 6.16 Notation adopted to describe the abnormal histology visualised in *gne2*.

To aid in the discussion of *gne2*, the following notation is used to describe the position but not the identity of the wall layers within the mature microsporangium. The position denoted as S1 is normally occupied by the sporocytes, S2 the tapetum, S3 the middle layer and S4 the endothecium. Additionally, S2/3 denotes the area normally occupied by the inner secondary parietal layer, the precursor of the tapetum and middle layer cells.

OSP is the outer secondary parietal layer and PS is the primary sporogenous cells.
**Fig. 6.17** Possible cell fates of the inner secondary parietal layer in *gne2*.

**a.** The inner secondary parietal layer (ISP), may not divide in *gne2*, and these cells may remain undifferentiated or adopt a sporogenous (S), Tapetal (Ta) or middle layer (Mi) fate. However, this would implicate a random distribution of Tapetal and Middle layer cells, which is not visualised in *gne2*.

**b.** Some cell division may occur in the inner secondary parietal layer resulting in the formation of isolated islands of Tapetal (Ta) and middle (Mi) layer cells. This would account for the cell pattern observed in the *gne2* microsporangium, whereby tapetal cells are only visualised adjacent to middle layer cells. Some inner secondary parietal layer may also remain undifferentiated or adopt a sporogenous (S) cell fate.
Possible cell fates of the ISP layer in *gne2*

a. No division of ISP cells
   - random Ta and Mi

b. Some division of ISP cells
   - islands of Ta and Mi
**Fig. 6.18** Possible explanations for the occurrence of sporogenous juxtaposed to endothecium cells in *gne2*.

**a.** A change in cell fate of the inner secondary parietal cells (S2/3) to sporogenous cells (S1) would result in some sporogenous cells developing next to endothecium cells.

**b.** Extra divisions in the sporogenous cells (S1) would also result in sporogenous cells adjacent to endothecium cells, providing the inner secondary parietal layer was incomplete in *gne2*.

**c.** Both the inner secondary parietal layer (ISP) and endothecium (En) originate from the division of the primary parietal layer. Therefore, the formation of a complete endothecium layer (En) present in *gne2*, implicates a complete inner secondary parietal (ISP) layer was also achieved. This observation opposes the hypothesis that extra divisions of the sporogenous cells results in these cell type lying adjacent to endothecium cells (**a.**) and favours the idea that the inner secondary parietal cells change their cellular fate to adopt sporogenous phenotype (**b.**).
b. S1 cell division

a. S2/3 → S1

c. Cell fate of the Primary parietal layer in *gne2.*
Complete En → complete ISP

**Diagram:**
- **S1**, **S2/3**, **S4**
- **PP**
- **OSP**
- **ISP**
- **En**
- **ISP**
Fig. 6.19 Possible nature of the inductive signal for the specification of pattern in the anther microsporangium.

a. An inductive signal required for the specification of pattern in the anther microsporangium may be generated or initiated by the archesporial (A) or sporogenous cells (S), and radially propagated to the surrounding cells, which perceive the signal relative to their position and differentiate accordingly.

b. This signal may be propagated as a diffusible morphogen that forms a concentration gradient radiating out from the centre of the microsporangium. Alternatively the signal may be laterally propagated from one cell to the next.
DIFUSIBLE MORPHOGEN?

LATERALLY PROPAGATED SIGNAL?
Table 6.1 Segregation analysis in A6-17 and A9-127.

Segregation analysis of kanamycin resistance in seedlings and GUS activity in the floral buds of two T3 transgenic *A. thaliana* lines, A6-17 and A9-127, showing a 3:1 segregation of the promoter-GUS transgenes present.
<table>
<thead>
<tr>
<th>T3 line</th>
<th>Seedlings grown on Kan</th>
<th>GUS activity in floral buds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kan&lt;sup&gt;res&lt;/sup&gt;</td>
<td>kan&lt;sup&gt;sen&lt;/sup&gt;</td>
</tr>
<tr>
<td>A6-17</td>
<td>314</td>
<td>96</td>
</tr>
<tr>
<td>A9-127</td>
<td>241</td>
<td>90</td>
</tr>
</tbody>
</table>
Table 6.2 Number of M2 families screened.

Number of $\gamma$-irradiated transgenic M2 families screened for GUS activity, indicating the total number of plants screened and the number of GUS negative families isolated. The numbers of GUS positive, male sterile families isolated during this screen are also shown.
<table>
<thead>
<tr>
<th>M2 plant line</th>
<th>No. of M2 families screened</th>
<th>No. of plants screened</th>
<th>No. of GUS negative families isolated [No. which are ms]</th>
<th>No. of GUS positive families isolated which are ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>A6-GUS</td>
<td>1241</td>
<td>11554</td>
<td>2 [2]</td>
<td>6</td>
</tr>
<tr>
<td>A9-GUS</td>
<td>1625</td>
<td>13821</td>
<td>5 [3]</td>
<td>18</td>
</tr>
</tbody>
</table>
Table 6.3 Cross pollination of A6-17 and A9-127 with three existing male sterile A. thaliana lines.

Analysis of crossings of A6 and A9-GUS with existing male sterile lines All data shown are male sterile F2 progeny, which PCR positive for the GUS gene, except the wild type which is male fertile and is PCR negative for the GUS gene.
<table>
<thead>
<tr>
<th>Plant genotype</th>
<th>plant No.</th>
<th>GUS activity (nmoles MU/min./mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ms37 /A6-17</td>
<td>14</td>
<td>259.6</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>282.5</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>316.5</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>559.9</td>
</tr>
<tr>
<td>ms33 /A9-127</td>
<td>20</td>
<td>401.6</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>293.9</td>
</tr>
<tr>
<td>ms32 /A6-17</td>
<td>24</td>
<td>235.3</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>76.0</td>
</tr>
<tr>
<td>ms32 /A9-127</td>
<td>28</td>
<td>58.6</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>43.6</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>26.8</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>198.4</td>
</tr>
<tr>
<td>WT C24</td>
<td>35</td>
<td>4.8</td>
</tr>
</tbody>
</table>
Table 6.4 Floral organ number in *gne7*.

Number of floral organs found in forty individual flowers harvested from homozygous *gne7* plants.
<table>
<thead>
<tr>
<th>Number of floral organs found in individual flowers</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Sepals</td>
</tr>
<tr>
<td>Petals</td>
</tr>
<tr>
<td>Stamens</td>
</tr>
<tr>
<td>Carpels</td>
</tr>
</tbody>
</table>


Table 6.5 Allelism test crosses between GUS negative mutants.

Allelism test crosses carried out between the phenotypically similar mutants. The presence of male-sterility in the progeny indicates the mutation has occurred within the same gene in both parental lines. Therefore $gne2$, $gne3$ and $gne5$ are allelic, but $gne1$ and $gne4$ are not.
<table>
<thead>
<tr>
<th>Allelism test cross</th>
<th>No. of male sterile progeny</th>
<th>No. of male fertile progeny</th>
<th>Approximate No. of F2 test cross progeny sown</th>
</tr>
</thead>
<tbody>
<tr>
<td>gn2 x gn3/C24</td>
<td>5</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>gn2 x gn5/pAF11</td>
<td>8</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>gn3 x gn5/pAF11</td>
<td>2</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>gn1 x gn4/C24</td>
<td>0</td>
<td>34</td>
<td>35</td>
</tr>
</tbody>
</table>
Table 6.6 Visible markers of the multimarker W100F.

Genetic map positions of the W100F visible markers on the *A. thaliana* integrated genetic map, and description of homozygous phenotypes displayed.
<table>
<thead>
<tr>
<th>Visible Marker</th>
<th>Chromosome - Map position (cM)</th>
<th>Homozygous phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>an-1</em> (angustifolia)</td>
<td>I - 10.5</td>
<td>Narrow leaves and twisted siliques</td>
</tr>
<tr>
<td><em>ap1-1</em> (apetela)</td>
<td>I - 98.7</td>
<td>No petals or rudimentary petals</td>
</tr>
<tr>
<td><em>er-1</em> petioles(erecta)</td>
<td>II - 56.5</td>
<td>Compact rosette, short and short stockey siliques</td>
</tr>
<tr>
<td><em>py-1</em> (pyrimidine requiring)</td>
<td>II - 61.9</td>
<td>Leaves except cotyledons pale/yellow</td>
</tr>
<tr>
<td><em>hy2-2</em> (long hypocotyl)</td>
<td>III - 8.5</td>
<td>Elongated hypocotyl</td>
</tr>
<tr>
<td><em>gl1-1</em> leaves(glabra)</td>
<td>III - 44.3</td>
<td>Trichomes absent on</td>
</tr>
<tr>
<td><em>bp-1</em> (brevipedicellus)</td>
<td>IV - 17.7</td>
<td>Short pedicels forcing siliques to point downwards</td>
</tr>
<tr>
<td><em>cer2-2</em> (eceriferum)</td>
<td>IV - 57</td>
<td>Stem bright green due to reduced wax layer</td>
</tr>
<tr>
<td><em>tt3-1</em> (transparent testa)</td>
<td>V - 61.5</td>
<td>yellow seeds</td>
</tr>
</tbody>
</table>
Table 6.7 Mapping data from F2 W100F/gnet lines.
Table 6.8 Mapping data from F2 W100F/gne5 lines.
Table 6.9 Chi2 analysis of W100F/gne5 mapping population.

Segregation analysis of the W100F markers and male sterility in the gne5/W100F F2 mapping population.
<table>
<thead>
<tr>
<th>Phenotypic Marker</th>
<th>No. of positive F2 plants</th>
<th>No. of negative F2 plants</th>
<th>Chi² value</th>
</tr>
</thead>
<tbody>
<tr>
<td>an-1</td>
<td>45</td>
<td>137</td>
<td>0.007</td>
</tr>
<tr>
<td>ap1-1</td>
<td>40</td>
<td>146</td>
<td>1.4</td>
</tr>
<tr>
<td>er-1</td>
<td>52</td>
<td>131</td>
<td>1.1</td>
</tr>
<tr>
<td>py-1</td>
<td>48</td>
<td>151</td>
<td>0.08</td>
</tr>
<tr>
<td>hy2-2</td>
<td>51</td>
<td>146</td>
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<tr>
<td>bp-1</td>
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<td>6.5</td>
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<tr>
<td>cer2-2</td>
<td>48</td>
<td>139</td>
<td>0.04</td>
</tr>
<tr>
<td>tt3-1</td>
<td>44</td>
<td>125</td>
<td>0.1</td>
</tr>
<tr>
<td>ms</td>
<td>54</td>
<td>131</td>
<td>1.7</td>
</tr>
</tbody>
</table>
Table 6.10 Chi$^2$ analysis of W100F/gne5 ms individuals.

Segregation analysis of W100F markers in the 54 ms individuals from the gne5/W100F F2 mapping population.
<table>
<thead>
<tr>
<th>Marker</th>
<th>No. of positive F2 plants</th>
<th>No. of negative F2 plants</th>
<th>Chi² value</th>
</tr>
</thead>
<tbody>
<tr>
<td>an-1</td>
<td>9</td>
<td>44</td>
<td>1.8</td>
</tr>
<tr>
<td>ap1-1</td>
<td>9</td>
<td>45</td>
<td>2</td>
</tr>
<tr>
<td>er-1</td>
<td>14</td>
<td>40</td>
<td>0.02</td>
</tr>
<tr>
<td>py-1</td>
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<td>14</td>
<td>40</td>
<td>0.02</td>
</tr>
<tr>
<td>cer2-2</td>
<td>13</td>
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<td>0.02</td>
</tr>
<tr>
<td>tt3-1</td>
<td>6</td>
<td>45</td>
<td>4.76</td>
</tr>
</tbody>
</table>
Table 6.11 Numbers of individual cell types present in a premeiotic anther cross section from WT A. thaliana and gne2.

Abbreviations: **S**, sporogenous cells; **Ta**, tapetal cells; **Mi**, middle layer cells; **En**, endothecium cells.

Comparison of sporogenous, middle layer and tapetal cell numbers suggests the inner secondary parietal cells, in gne2, have failed to undergo the normal division to tapetal and middle layer cells.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell numbers in WT from Fig. 6.5.b</th>
<th>Cell numbers in gne2 from Fig. 6.7.f</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Ta</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>Mi</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>En</td>
<td>15</td>
<td>16</td>
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</tbody>
</table>
Chapter 7
Overview
7.1 Genetic analysis of stamen initiation and development in
A. thaliana

Through the analysis of floral mutants of A. thaliana, a number of genes have been identified which are involved in the genetic regulation of floral development (Okada and Shimura, 1994). A cascade of genetic signalling, initiated in the vegetative meristem and triggered by environmental and endogenous signals, cause the transition of the vegetative meristem to an inflorescence meristem. Subsequently, the floral meristem identity genes (1.1.2) are expressed within the floral primordia or meristematic cells which grow out from the inflorescence meristem. The floral meristem identity genes promote the expression of the floral homeotic genes (1.1.1), which become delimited to specific cell types of the floral primordia by the control of the cadastral genes (1.1.3). These genes control the boundaries of floral homeotic gene action. As a consequence of individual or overlapping expression domains of the floral homeotic genes within the primordia cells, the four distinct types of floral organs, the sepals, petals, stamens and carpels are specified to develop within the flower in their predetermined position.

The six stamens of A. thaliana develop in a whorl that surrounds the central gynoecium in the WT flower, specified by the expression of the floral homeotic genes AP3 and AG. These two genes are restricted to the stamen primordial cells by the action of three cadastral genes SUP, AP2 and LUG. The SUP gene is expressed in a subset of the AP3 expressing cells such that SUP gene domain defines a border between the third whorl stamens and the fourth whorl carpels (Sakai et al., 1995). The LUG gene prevents the ectopic expression of AP3 in the first whorl sepals or AG in the first whorl sepals or second whorls petals, aided by the AP2 gene which is the main antagonist of AG (Lui and Meyerowitz, 1995).

Both the AP3 and AG genes encode transcription factors that contain a MADS box domain (Schwarz-Sommer et al., 1990), which functions in DNA binding (Yanofsky et al., 1990, Jack et al., 1992, Mandel et al., 1992, Goto & Meyerowitz, 1994). It is likely therefore that the development of the stamen is sustained through a promotive cascade of gene regulation via these transcription factors which promote the growth and differentiation of the stamen primordial cells, although no such candidate, subordinate genes have been identified as yet. However, a number of genes have been isolated through the differential screening of anther specific libraries (Scott et al., 1991; Koltunow et al., 1990; 1.3.2), many of which
show localised expression within the tapetum, and in addition a number of later acting genes have been identified which when mutated disrupt the normal progression of microsporogenesis and lead to male sterility (1.4).

Utilising a transgenic screening approach of a $\gamma$-irradiated *A. thaliana* population, the work described in this thesis has focused on bridging the gap between the expression of *AP3/AG* in the initiation of stamen development and the expression of the tapetal-specific genes, *A6* and *A9*. To allow the identification and characterisation of genes involved in stamen tissue histospecification. The ectopic effect of the late expression of the *AG* gene in the tapetum of *A. thaliana* has also been investigated, as well as a 5’ deletion analysis of the A9 promoter.

**7.2 Investigation of ectopic *AG* expression in *A. thaliana* tapetal cells**

The *AG* gene controls the specification of the stamens and carpels in the third and fourth whorls respectively, as well as controlling the determinacy of the flower. *AG* also acts as a negative regulator of the *AP2* gene, within the developing floral meristem (Drews et al., 1991). The generation of a tagged mutant allele of the *AG* gene, *ag-2* (Feldmann et al., 1989), allowed the cloning of the *AG* coding sequence, which was found to contain a putative DNA binding and dimerisation domain, and shared sequence homology with other known transcription factors (Yanofsky et al., 1990).

*In situ* hybridisation studies using an antisense *AG* RNA probe in WT floral primordia, show *AG* RNA is restricted to the third and forth whorls and is first detected after the emergence of the sepal primordia. The detection of *AG* RNA increases to a strong uniform signal throughout the third and fourth whorls by the time the anther and filament have become distinct and the carpel primordia have emerged (Drews et al., 1991). Later during meiosis in the sporogenous cells, *AG* mRNA becomes restricted to specific cell types of the anther, detected at highest levels in the connective tissue, with a lower signal seen in the filament and the anther cell walls. *AG* mRNA is still detected in the anther cell walls prior to dehiscence, which at this stage are composed of only the epidermis and the endothecium (Bowman et al., 1991).

In order to further investigate the role of *AG* in late stamen differentiation, the *AG* coding region was isolated by PCR from WT L. *erecta*, and fused downstream of two different tapetal-specific promoters, *A6* and *A9*, in both the sense and antisense orientations. These two promoters are active in the tapetum of the anther.
wall, during meiosis of the sporogenous cells and up until the formation of the microspores, when the tapetum begins to degenerate. Consequently, these promoter activities almost completely coincide with detection of WT AG mRNA in the anther cell walls (Bowman et al., 1991). 15 A6-AG and 12 A9-AG antisense, as well as 1 A6-AG and 1 A9-AG sense A. thaliana transformants were generated. T2 seed from each transformant were grown and floral buds were examined under the light microscopy for any abnormalities in the floral organs. In both the antisense and 2 sense transgenic lines the stamens possessed a WT morphology, and seed set was not affected.

The promoters utilised were strong enough to elicit a change in the abundance of native AG mRNA levels. In the antisense transgene, 2 bp errors had occurred in PCR amplification of the AG gene, which were not likely to cause a problem for antisense analysis. Therefore, lack of any visible alterations in these transgenic lines may indicate that AG has no function in tapetal development at this late stage of cellular differentiation. Alternatively, the introduced transgenes may have failed to work. An inadequacy in experimental design is that it was impossible to determine whether or not the mRNA levels solely in the tapetum had been altered by the introduced transgenes.

7.3 Characterisation of the A9 promoter in N. tabacum and A. thaliana

In order to further characterise the A9 promoter and identify sequence important for the transcriptional activity of the A9 gene, five 5’ deletion fragments of the A9 promoter less than 329 bp in length, were cloned upstream of the LUC gene into pBin19 binary vectors. The resulting plasmids, pAF77, pAF76, pAF75, pAF83 and pAF86 contained 312, 232,178,130 and 65 bp respectively of 5’ untranslated sequence of the A9 gene which were transformed into A. thaliana and N. tabacum using A. tumefaciens. Despite two N. tabacum and one A. thaliana transformation no pAF75 transformants were recovered. This may have been due to a failure of the kanamycin gene to select for regenerating transgenic plant material.

In both N. tabacum and A. thaliana transformants pAF1, pAF77 and pAF76 transformants, containing 934, 312 and 232 bp of 5’ A9 promoter sequence, consistently give a LUC activities averaging in the millions of units per μg protein, while pAF83 and pAF86 transformants, with 130 and 65 bp of A9 sequence, give LUC activities of less than 250 units per μg protein. Therefore the essential 5’ untranslated region of the A9 promoter required for gene transcription can be
deduced to lie between 130 and 232 bp upstream of the ATG start site. No transformants or WT control plants showed LUC activity in other floral organs or sporophytic tissue.

However, LUC activity was detected in the pollen of all *N. tabacum* A9-LUC transformants. Although LUC activity in the pollen of pAF1 *N. tabacum* transformants was over forty times lower than that detected in the anthers, expression in the pollen showed a small but notable increase through successive 5' deletions of the A9 promoter. No LUC activity was detected in the 930 bp A9-LUC *A. thaliana* transformant. However, two of the 5' A9 deletion-LUC *A. thaliana* transformants, pAF83 and pAF86, did show low LUC activity in the pollen. The 65 bp A9 promoter fragment in *A. thaliana* also showed higher pollen expression than the 130 bp A9 fragment. A9-GUS *N. tabacum* transformants, containing a 930 bp promoter fragment, also showed the histochemical detection of the *GUS* gene in pollen extracts (data not shown). Subsequently, RT PCR analysis in *B. napus* showed that A9 is expressed in the buds, but is not expressed in the pollen.

Previously, the validity of *GUS* expression in *N. tabacum* pollen has been questioned (Uknes et al., 1993). Uknes and coworkers demonstrated that although a PR1a- *GUS* transgene was expressed in *N. tabacum* pollen, the endogenous *PR1a* transcript was not detected in pollen extracts. These experiments concluded that the *GUS* coding region specifically influences the expression of the transgene in *N. tabacum* pollen causing ectopic expression. These implications may be applicable to the *LUC* gene, perhaps causing low pollen expression in *N. tabacum*. To be applicable in this work, the explanation by Uknes and coworkers would have to extend to *A. thaliana*. As only two of the five transgenes showed pollen expression in *A. thaliana*, these observations tend to contradict that *LUC* would cause ectopic pollen expression.

The activity of the *A. thaliana*-derived, 930 bp A9 promoter in *N. tabacum* pollen, but not *B. napus* pollen may be explained by *A. thaliana* being more closely related to *B. napus* than to *N. tabacum*. Additionally, the pollen development in these two species is different. Mature pollen from *B. napus* is trinucleate, while *N. tabacum* pollen grains remains dinucleate until after germination. It may be that the *A. thaliana* promoter sequences are interpreted differently in *N. tabacum*, leading to A9 activity in the pollen. The spurious activity of the 5' A9 promoter deletion fragments in *A. thaliana* pollen is most likely as a result of the loss of upstream regions, which may be negative regulators, the deletion of
which result in pollen expression. If _N. tabacum_ does not fully recognise these specific sequences, contained between 930 and 130 bp upstream of the promoters' ATG start site, then this would explain why low pollen activity is detected in all A9- _LUC_ transformants, and why pollen activity increases through successive deletions.

7.4 Evaluation of the A6 and A9 tapetal-specific promoters to define and delimit gene expression along the stamen differentiation pathway

The main achievement of this thesis, has been to identify mutants of _A. thaliana_ that are affected in the specification or differentiation of specialised anther cell types. The strategy adopted was to screen a transgenic, mutagenised population of _A. thaliana_ for the down regulation of either A6 or A9 tapetum-specific promoter-reporter chimaeric transgenes, indicating that a lesion had occurred upstream of the expression of these two promoters, along the stamen differentiation pathway.

In an original strategy it was perceived that the A9 and A6 promoters would provide consistent temporal windows around meiosis. Whereby the A9 promoter activity would delimit the start of meiosis and A6 the end. A number of single and double, chimaeric promoter-reporter gene fusions, utilising the coding regions of either the _LUC_ or _GUS_ reporter genes, were constructed and transformed into _N. tabacum_ and _A. thaliana_. The linear relationship between bud and anther length and developmental stage seen in _B. napus_ and _N. tabacum_ (Scott et al., 1991b; Paul et al., 1992; Hird et al., 1993) was extrapolated and confirmed in _A. thaliana_. Therefore, it was possible to ascertain if the temporal expression patterns of A6 and A9 driving either reporter gene would be compatible with our proposal, simply by assaying sized buds or anthers of relevant transgenic lines.

In the single A6 and A9- _LUC N. tabacum_ transformants, A9 activity was initially detected in 7 to 8 mm buds, around the start of meiosis as expected, and A6 activity also peaked at 10 mm around microspore release as previously documented. However, A6 activity was detected at a much lower level in buds as early as 5 mm and therefore strict temporal windows were not obtained. Additionally, in _A. thaliana_ single promoter-_GUS_ transformants the A6 promoter was activated prior to A9. In _N. tabacum_ and _A. thaliana_ transformants expressing double promoter-reporter chimaeric transgenes, varied patterns of A6 and A9 promoter activity were visualised. Some transformants showed A6 activity first,
while other lines showed simultaneous A6 and A9 activity. In a further subset of transgenic *N. tabacum* double transformants the expected temporal expression patterns of A6 and A9 were visualised. However, analysis of their progeny indicated that these patterns were not inherited, and could not therefore be relied upon to enable the accurate dissection of mutants into their allocated temporal windows.

The varied temporal patterns of activity of the A6 and A9 promoters in both transgenic *A. thaliana* and *N. tabacum* plants lines may have been due to either or both the positional effect of T-DNA insertion or the environmental plant growth conditions. Additionally, the *GUS* and *LUC* reporter genes may not have be sensitive or accurate enough to define the short temporal window around meiosis. Therefore, the application of A6 and A9 to delimit temporal windows along the stamen differentiation pathway was discontinued.

A third reporter gene, *TMS2*, was assessed to devise a visual screen to detect for the down regulation of A6 activity. The A6 promoter was fused upstream of the *TMS2* gene coding region and transformed into *A. thaliana*. The *TMS2* gene encodes amino hydrolase (*iaaH*) which converts biologically inactive auxin amides into active auxins, which are toxic to plants at elevated concentrations. Transgenic plant lines when sprayed with IAM did not produce any visually deformed anthers or flowers, as would be expected by any catalytic conversion of the IAM to a toxic IAA byproduct. However, the tapetum in these transgenic lines were not examined. Even if the tapetal cells did show abnormalities as a result of *TMS2* gene expression, such a system would not provide a suitable visual as proposed in our screening strategy due to the enclosed nature of these cell types. It may have be the case that a more toxic substrate such as NAM would have resulted in an abnormal floral phenotype. If a suitable A6-*TMS2* line had been generated, it is possible that this line at best would provided the early deterioration of the tapetum, most likely resulting in male sterility. With hindsight, such a line would have been futile in screening our M2 population, as all the genuine mutants isolated were ms anyway and would not have shown any difference in a visual screen to non-mutant plants.

### 7.5 Mutants of *A. thaliana* isolated, affected in stamen development

Homozygous A6-*GUS* and A9-*GUS* transgenic *A. thaliana* seed were exposed to 30 Krads of Cs$^{137}$, a *γ*-irradiation mutagenic agent to generate a M1 population. M2 plants were screened for the down regulation of tapetal-specific
promoter activity by assaying the flower buds for the lack or reduction in GUS activity. 8 GUS negative mutant lines were isolated, designated \textit{gne1} to \textit{gne8} respectively. \textit{gne6} and \textit{gne8} were male fertile, while the remaining six were ms. All the GUS negative lines showed a complete down regulation of the \textit{GUS} gene, except \textit{gne7} where 1 to 2 % of WT GUS activity was detected.

All the GUS negative mutants were out crossed to the W100F multimarker line for mapping analysis. F1 progeny were assayed for GUS activity to test for the integrity of the transgene. Unfortunately neither male fertile GUS negative lines, \textit{gne6} and \textit{gne8}, showed any GUS activity in their floral buds. Therefore, the lesion had occurred in the promoter-reporter transgene and was not a genuine down regulation of the GUS gene. No further analysis was carried out on \textit{gne6} and \textit{gne8}. Of the remaining ms GUS negative lines, only one line \textit{gne7}, showed any abnormal phenotypic characteristics other than male sterility. Homozygous \textit{gne7} mutants were smaller in stature than heterozygous plants and also showed defects in floral morphology, where abnormal and varied numbers of petals, stamens and carpels were visualised on floral inflorescences.

The genetic characterisation of the GUS negative lines was initiated with histological observations on the developing anther microsporangia to establish the nature of the lesion for each mutant. In WT \textit{A. thaliana} microsporogenesis follow a tightly regulated series of events, involving the synchronous differentiation of the four locules of the anther, which can be delimited by cytological landmark events. From observations of microsporogenesis it became apparent that these mutants fell into three distinct classes. The \textit{gne1} class comprising \textit{gne1} and \textit{gne4}; the \textit{gne2} class comprising \textit{gne2}, \textit{gne3} and \textit{gne5} and a third class consisting of only \textit{gne7}. Subsequent allelic test crosses between the similar GUS negative classes confirmed that \textit{gne2}, \textit{gne3} and \textit{gne5} are allelic, and that \textit{gne1} and \textit{gne4} are non-allelic. Therefore from the original 8 GUS negative mutant lines isolated, four non-allelic, ms mutants were confirmed, namely the \textit{gne1}, \textit{gne2}, \textit{gne4} and \textit{gne7} class. For discussion purposes \textit{gne1} and \textit{gne4} are grouped together because they confer a similar phenotype and may act in the same area of development. Map positions were investigated for these four mutant genes using the W100F multimarker tester line and CAPS analysis.

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7.5.1 *Arabidopsis thaliana* lines disrupted in tapetal differentiation - the *gne* mutant class

In the *gne* class, consisting of the non-allelic *gne* and *gne4* mutants, the initial differentiation of the microsporangia is normal. The four cell layers of the anther wall are clearly visible as single layers, concentrically arranged around the core of morphologically typical meiocytes, as in WT development. However, prior to meiosis the tapetal and middle layer cells progressively enlarge in size and develop a grossly enlarged single vacuole. The meiocytes in this mutant class do enter meiosis, and callose is visualised in the anther locule. Tetrads of microspores are also produced. The abnormal tetrads, middle layer and tapetum prematurely degenerate leaving an empty locule within the pre-dehiscent anther. Analysis of the tapetal cells in the *gne* using T.E.M show that these cell types possess a less dense cytoplasm, than WT counterparts, with a reduction in ribosome density, endoplasmic reticulum and other functional bodies.

An accurate genetic map position for the *gne* gene, was ascertained by the analysis of data gathered from *gne* x W100F, F2 recombinant mapping populations. From over 1500 F2 individuals grown, no recombinant individual was scored for both the *cer2* mutation and male sterility. This indicates the very close linkage of the *gne* locus to the visible marker *cer2*, which maps to 57.0 cM on the integrated-IV chromosome map. Therefore, the *gne* locus is located on chromosome IV, close to 57.0 cM.

Although a number of *gne4*/W100F mapping populations were grown and analysed, no linkage to *gne4* could be established, as neither male sterility nor the phenotypic markers from W100F showed a clear 3:1 segregation in the F2 population. In addition, CAPS mapping was incomplete, although a number of ms *gne4*/W100F F2 individuals were harvested and stored at -20 °C for such an analysis.

7.5.1.1 Role of the *gne* gene class

The mutation responsible for the lesion seen in the *gne* class, indicates that the WT gene products affected disrupts the normal differentiation of the tapetal and middle layer cells. The WT **GNE1** and **GNE4** genes, may normally be expressed in the tapetal and middle cell layers prior to meiosis, and may have a function in cell differentiation. Alternatively, these genes may be required for a metabolic pathway important to both these cell types.
If the cell lineage of the tapetal and middle layer cells originate from a common precursor, the inner secondary parietal cells, the \textit{gne1} class of mutation could affect the differentiation of this progenitor cell type. However, the microsporangium fate map of \textit{A. thaliana} would have to resemble the cell lineages seen in typical monocotyledenous plant species, such as \textit{Triticale} rather than normal dicotyledenous development. In typical dicotyledenous differentiation, such as \textit{N. tabacum}, the tapetal cells are derived from the inner secondary parietal cells, and the middle layer is derived from the outer secondary parietal cells. Mutations in the \textit{gne1} and \textit{gne4} genes are non-allelic, so although these two mutations cause similar phenotypes, they are not a consequence of a lesion in the same gene. Therefore \textit{gne1} and \textit{gne4} gene expression may show similar or close temporal patterns of expression, or even be individual components of the same regulatory pathway.

### 7.5.1.2 Future work for the \textit{gne1} and \textit{gne4} mutant lines

The proposed strategy for the isolation of the \textit{gne1} gene, has been to use a targeted tagging approach. The local transposition events of a nearby maize transposable element system could be used to cause selected insertional mutagenesis. A suitable homozygous \textit{A. thaliana} line, containing an \textit{I/dSpm} transposon on chromosome IV in the vicinity of the \textit{CER2} gene, has been crossed to a homozygous \textit{gne1} plant. The strategy now is to screen F1 progeny for revertant ms sectors, indicating the transposition of the mobile \textit{I} element into the \textit{GNE1} gene. The transposon can then be used as a genetic tag to isolate the bordering \textit{GNE1} sequence using IPCR. The partial \textit{GNE1} clone would then be used as a probe to screen WT genomic libraries to facilitating the isolation of the entire \textit{gne1} gene.

\textit{gne4} requires further analysis to ascertain a map position for this mutant, which will then direct a strategy for the isolation of the WT \textit{GNE4} gene. Therefore, long term future work for \textit{gne1} and \textit{gne4}, once the WT genes have been cloned will be analysis of gene function through gene expression patterns, and DNA and protein sequence analysis.

### 7.5.2 Mutants of \textit{A. thaliana} showing abnormal cell specification in the stamen microsporangium - the \textit{gne2} mutant class

In the \textit{gne2} class, consisting of three allelic mutant lines, \textit{gne2}, \textit{gne3} and
gne5, observations of anther transverse sections show that the histology of the microsporangia is aberrant from the earliest detectable stage. The anther wall in the gne2 mutant class consists of only two or three cell layers, with only the two outermost cell types, the presumptive epidermis and endothecium, forming continuous layers. In addition, the meiocytes present within the locule of gne2, are more numerous than those found in a WT anther of a similar developmental stage. Therefore, isolated islands of presumptive tapetal and middle layer cells are formed. So in some sections of the anther transverse section, the normal constitution of four cell layers is found in the microsporangium and in other places the meiocytes are seen developing adjacent to presumptive endothecium or middle layer cells.

The meiocytes present in the gne2 class initiate meiosis as callose is secreted. The tapetal cells become abnormally enlarged and vacuolate. Some of the meiocytes start to degenerate prior to the completion of meiosis. However, some of the meiocytes complete meiosis, but degenerate prior to cytokinesis, indicated by the presence of tetrads of microspores with cross walls and individual nuclei. The meiocytes, tetrads and tapetum prematurely degenerate in the gne2 mutant class, leaving the locules completely empty.

An approximate genetic map position was ascertained for the gne2 mutant class using both W100F and CAPS analysis. The analysis of data from an allelic gne5/W100F F2 mapping population, demonstrated linkage between male sterility and the tt3-1 phenotypic marker, assigning the gne2 gene class to chromosome V. Using the Kosambi mapping formula and calculating the standard deviation, the gne5 mutation was calculated to maps at a distance of 37.6 +/- 11.5 cM from the tt3-1 marker on chromosome V. The tt3-1 locus, maps to 61.5 cM on the Integrated-V chromosome map. Using CAPS analysis on F2 gne2/W100F and gne3/W100F ms individuals, evaluation of the recombination fraction between for the ASA1 CAPS marker, indicated that the gne2 lesion maps to a distance of 1.8 cM +/- 1.3 cM from the ASA1 marker, while gne3/ASA1 CAPS data indicates a map distance of 21.18 +/- 5.7 cM from the ASA1 marker. The ASA1 marker maps to 14.8 cM on the Integrated-V chromosome map. Therefore, the GNE2 gene is located on chromosome V, in the proximity of the ASA1 marker.

7.5.2.1 Possible role of the gne2 gene class

From the earliest detectable stage in the gne2 mutant class, the
specification of specific anther cell types within the microsporangium is disrupted. Observation of the microsporangia in the \textit{gne2} mutant class, show a cylindrical asymmetric core, enclosed by the endothecium, that is composed of islands of tapetal and middle layer cells and numerous meiocytes. Presuming that the tapetal and middle layer cells have a common precursor, the inner secondary parietal cells (as previously postulated from the mutation seen in the \textit{gne1} mutant class) the \textit{GNE2} gene may be initially expressed in the inner secondary parietal cells. \textit{GNE2} may play a role in the assignment or interpretation of cell fate in the subsequent formative division of the inner secondary parietal cells to the tapetal and middle layer cells. The \textit{GNE2} gene may be involved in signalling events specifying patterning information or may be involved in the formation of a boundary between the sporogenous and tapetal and middle layer directing the differential development of these cell types.

### 7.5.2.2 Possible mechanism of \textit{gne2} action

To hypothesise on the possible mechanism by which the \textit{gne2} gene may control cell specific differentiation in the anther, an examination of the existing knowledge of cell signalling and pattern specification in other plant tissue types, and animal species was undertaken. One possibility might be that an inductive signal originates from the sporogenous cells and is radially propagated outwards specifying patterning information within the microsporangium. This signal may specify a primary cell state, directing all the microsporangium cells to differentiate as sporogenous cells. The WT GNE2 product may act to repress this signal and direct tapetal and middle layer cell fate. In a simple scenario, presuming \textit{gne2} is a null mutation, a complete transformation of tapetal and middle layer cells to sporogenous cells would be expected. However, some presumptive tapetal and middle layer cells are seen, possibly explained by the occurrence of some division of the inner secondary parietal cells to tapetal and middle layer cells.

Alternatively the \textit{gne2} mutation may be involved in the formation of boundaries. The formation of boundaries has been implicated in the differential development of equipotential cells in floral development and the \textit{gne2} mutation demonstrates similarities to the \textit{sup} mutation. In \textit{sup} mutants, the stamens extend beyond the third whorl at the expense of the carpels in the fourth whorl. Similarly, the \textit{gne2} mutation appears to cause extra sporogenous cells at the expense of the tapetal and middle layer cells, which may explain the islands of tapetal and middle
layer cells that are visualised in the *gne2* class of mutants.

In both vertebrate and invertebrate pattern specification, diffusible morphogenic factors have shown to be responsible for compartmentation and boundary formation within equipotential cells. Therefore, the *GNE2* gene may be either directly or indirectly involved in the regulation of cell boundaries between the sporogenous cells and tapetal and middle layer cells. The *GNE2* gene product may establish the tapetal and middle layer cells to form a single compartment and another gene product coupled to *GNE2* expression may be required for the differentiation between the tapetal and middle layer cells. Alternatively, expression of *GNE2* in the tapetal cells may establish two boundaries, one between the sporogenous cells and the tapetum and one between the tapetal and middle layer cells. Inductive signalling across these boundaries present in the microsporangium may be required for the initiation of *GNE2* gene action to maintain such boundaries. In either case, the *gne2* mutation would cause the loss of boundaries between the sporogenous and tapetal and middle layer cells, causing abnormal patterning in the microsporangium.

### 7.5.2.3 Future work concerning *gne2*

Fine mapping of the *gne2* locus should enable the isolation of the *GNE2* gene by map based cloning techniques. The future genetic analysis of *GNE2* will enable the elucidation of the temporal and spatial patterns of expression of this gene. Additionally, DNA and protein sequence analysis may demonstrate indicative motifs, giving an insight to the functional nature of the GNE2 protein. Therefore, future analysis will confirm or discredit the speculation that *GNE2* plays a role in patterning information in the WT anther.

### 7.5.3 A mutant showing asynchronous microgametogenesis - *gne7* - and the close linkage of this mutant line to the physical map position of the *PHYC* gene

In WT floral buds of *A. thaliana*, the formation of both the male and female gametes follows a tightly regulated, synchronous sequence of events that shows a direct linear relationship to bud length. In the *gne7* mutant, observation of a sequential series of floral bud transverse sections, shows microgametogenesis but not megagametogenesis, displays an asynchronous and abnormal pattern of histodifferentiation. In *gne7* meiosis occurs asynchronously between adjacent
anther locules. However, meiosis is completed in the mutant line resulting in free microspore which proceed to develop an exine coat. Shortly after this time the microspores start to degenerate resulting in premature pollen abortion and male sterility.

An accurate genetic map position for the gne7 mutation was attained from the CAPS analysis of gne7/W100F F2 ms individuals, using the ASA1 and PHYC molecular markers. Recombination of the ASA1 marker, in these individuals, gave a map distance of 29.8 cM +/- 6.9 cM from the ASA1 locus on chromosome V. In PHYC mapping experiments, using the same DNA samples, the expected 2 Kb DNA PCR fragment was not synthesised from any of the recombinant individuals. The expected 2 kb band was detected in control homozygous C24 and L. erecta parental lines, indicating that the gne7 mutant contained a deletion spanning the 2 Kb PHYC locus.

Analysis of the size of the deletion using a 14 kb λ clone in southern analysis, initially indicated the entire deletion of this clone in gne7. However, subsequent analysis using the 5' end of this clone indicated part of this fragment to be present in gne7. To enable the complementation of the gne7 phenotype and the isolation of genes responsible for male sterility, a BamHI fragment of the PHYC gene excised from the λ 5.2 clone was used to probe a λTI2-plasmid-convertable phage cDNA library (Fuse et al., 1995), however due to time limits the desired cDNA clone was not isolated.

Subsequent analysis by Devlin and coworkers were able to show a separation between the PHYC gene and male sterility seen in gne7. Transgenic pollen from a homozygous 35S-PHYC sense A. thaliana line was used to pollinate a ms gne7 individual, and 100 F2 seeds were germinated. PCR analysis ascertained that 18 of the 24 gne7 F2 plants had the 35S-PHYC transgene present, demonstrating that the 35S-PHYC transgene does not rescue the gne7 phenotype. Therefore, other gene(s) deleted in close proximity of the PHYC gene are responsible for the reduced stature, floral abnormalities and male sterility seen in gne7.

7.5.3.1 Role of the gne7 gene

The asynchronous initiation of meiosis demonstrated in gne7, implicates a genetic lesion which has a WT function in the regulatory mechanism involved in the imposition of synchrony in the meiocytes. In support of this proposition, is the
observation that the synchronous initiation of meiosis in WT development, is achieved by some factor(s), that originate from outside the sporogenous cells (Walters, 1985). Additionally, Ito and Stern, 1967, concluded that the meiocytes are only capable of developing into mature pollen grains, in vivo, without the influence of the tapetal cells, if they have already reached the leptotene-zygotene stage of meiosis.

The control of meiotic synchrony is thought to be imposed by the accumulation of the sporogenous cells and slightly later the tapetal cells, at the G1 stage of meiotic interphase. Both cell types are then simultaneously released from this hold entering DNA synthesis together. The factor(s) responsible for the accumulation and release of these two cell types from the G1 stage, are proposed to act via plasmodesmata connecting the tapetal cells to the meiocytes, creating a syncytium through which the regulatory molecule(s) can freely pass (Heslop-Harrison, 1966a, 1966b). Therefore one of the mutations in gne7 may disrupt one aspect of the pathway responsible for the synthesis of such a hypothesised factor, and could encode an enzyme responsible for the synthesis of precursors or the factor itself.

7.5.3.2 Future work concerning gne7

The proposed strategy for isolation of the genes deleted in gne7 is to use a BamHI fragment of the PHYC gene, obtained from a λ 5.2 clone, to screen a Ti-plasmid-convertable λ phage library (Fuse et al., 1995) and isolate the PHYC containing clone. This PHYC clone can then be directly converted into a binary vector suitable for complementation analysis of the gne7 line. If the complementation of gne7 is complete, then digestion and complementation of gne7 with individual fragments of the λ.Ti2 PHYC clone should enable the genetic dissection of the different phenotypes seen in gne7. Once cloned, the further analysis of promoter(s) and gene(s) identified should enable an insight into the control of synchrony during microsporogenesis, and perhaps other aspects of floral development.

7.6 Summary

The thesis describes a transgenic screening approach which was utilised to identify mutants of A. thaliana affected in stamen development. Transgenic A. thaliana seed, homozygous for either an A6-GUS or A9-GUS transgene were
exposed to a Cs137 source of \(\gamma\)-irradiation. The resulting M2 families were screened for the down regulation of A6 or A9 promoter activity, by assaying for the absence of GUS activity in the floral buds. Four non-allelic ms mutant lines, \textit{gne1}, \textit{gne2}, \textit{gne4} and \textit{gne7}, were identified. The first class \textit{gne1}, encompasses the non-allelic \textit{gne4} mutant. The WT gene products mutagenised in these lines are implicated in the control of differentiation of the tapetal and middle layer cells. The \textit{gne1} mutation has been mapped to 57.0 cM on the integrated-IV chromosome map. The second mutant class \textit{gne2} maps in the region of the ASA1 marker on the chromosome V. The \textit{GNE2} product may be involved in the control of patterning information in the microsporangium, and may play a role in the assignment or interpretation of the tapetal and middle layer cell fate. The third mutant identified, \textit{gne7} contains a large deletion spanning the \textit{PHYC} locus on chromosome V. \textit{gne7} results in the asynchronous progression of meiosis. The \textit{gne7} mutant also shows a reduction in height and increase in branching of the inflorescence meristem, as well as abnormal numbers of floral organs. The deletion of the \textit{PHYC} gene has been shown not to be responsible for the \textit{gne7} phenotype. Therefore, other deleted genes, in close proximity of \textit{PHYC} locus are implicated in the abnormalities visualised in this mutant line.

An original screening strategy was designed to assign the GUS negative mutants isolated to temporal windows along the stamen differentiation pathway. However, the A6 and A9 promoters were unable to demonstrate consistent, heritable patterns of expression in the plant lines generated. Another approach examined was the development of a visual screen to isolate stamen development mutants utilising a A6-\textit{TMS2} transgene. The \textit{TMS2} gene encodes an amino hydrolase which converts inactive auxin compounds to toxic auxins. Spraying of transgenic \textit{A. thaliana} lines generated with a IAM substrate did not cause any defects in floral morphology as hoped. Therefore this strategy was also rejected.

Other work in this thesis examines the sense and antisense expression of the \textit{AG} coding sequence in the tapetum, under the control of both the A6 or A9 promoters. No abnormal floral phenotypes were visualised in the transgenic lines generated which was unable to demonstrate the late role of the \textit{AG} gene in this tissue type. Additionally, characterisation of the A9 promoter by 5' deletion analysis indicated that essential regions of promoter sequence required for the transcriptional control lie between 130 and 232 bp upstream of the ATG start site of the A9 gene.
Appendix

LUC activities detected in the floral buds of A9 (deletion fragments)-LUC N. tabacum transformants and WT SR1 plants
Table 1 LUC assays on anther and pollen extracts from pAF1 *N. tabacum* transformants - 930 bp A9 promoter fragment

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Table 2 LUC assays on anther and pollen extracts from pAF77 *N. tabacum* transformants - 312 bp A9 promoter fragment

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| 12              | 947755                          | 18              | 8151585                         |
| 14              | 252636                          | 27              | 840                             |
| 15              | 2016401                         | 31              | 1253                            |
| 16              | 2762828                         | 43              | 4700                            |
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| pAF77-3         |                                 | pAF77-11        |                                 |
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| 9               | 1114091                         | 10              | 3855953                         |
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| 10              | 695336                          | 12              | 19663864                        |
| 10              | 795496                          | 14              | 8859952                         |
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| 10.5            | 719600                          | 23              | 41790                           |
| 11              | 785980                          | 28              | 1646                            |
| 12.5            | 499613                          | 42              | 1716                            |
| pollen          | 324665                          | pollen          | 159555                          |

| pAF77-4         |                                 | pAF77-12        |                                 |
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Table 3 LUC assays on anther and pollen extracts from pAF76 *N. tabacum* transformants - 232 bp A9 promoter fragment

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Table 5 LUC assays on anther and pollen extracts from pAF86 *N. tabacum* transformants - 65 bp A9 promoter fragment

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| **9**          | **68**                        |
| 11             | 109                           |
| 12             | 181                           |
| 14             | 80                            |
| 15             | 57                            |
| 33             | 17                            |
| 46             | 180                           |
| pollen         | 738                           |
Table 6 LUC assays on anther, pollen and sporophytic tissue extracts from WT SRI N. tabacum plants

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Haldane, J.B.S. (1919). The combination of linkage values and the calculation of distances between the loci of linked factors. J. Genet. 8, 299-309.


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