# MALE CONE DEVELOPMENT IN *PINUS RADIATA*

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# ABSTRACT

Light microscopy and transmission electron microscopy were used to investigate the morphological, anatomical changes and the timing of these changes during male cone development of *Pinus radiata* growing in the central part of the North Island, New Zealand. The timing of developmental events, including the initiation of the male cone primordia, the onset of meiosis of pollen mother cells and the formation of pollen grains were recorded. Their relationship with environmental factors in comparison with pine species growing in the Northern Hemisphere was discussed.

Some significant morphological aspects of male cone buds, microsporophylls and structural/ultrastructural changes of microsporangia, tapetal cells and pollen mother cells during the meiotic processes in particular, were reported in the morphological and anatomical study.

In correlation with these structural/ultrastructrual changes, the soluble protein content, banding patterns of the total soluble protein, banding patterns of four isoenzymes during male cone development were studied by SDS-PAGE and isoelectric focusing techniques. Seven soluble protein species were detected by SDS-PAGE closely related to the different developmental stages of the male cone, and one of them with a molecular mass of 20.5 KD in particular was found to be a potential male cone tissue specific gene expression product. Acid phosphatase, esterase, malate dehydrogenase and peroxidase were studied during male cone development, using isoelectric focusing methodology. Variations in banding patterns of the enzyme activity and number of isoforms of each enzyme in relation to the different developmental stages of the male cone were revealed. A number of isoforms of these four isoenzymes were found to be unique to specific developmental stages.

A search for floral-specific genes controlling floral developmental events was attempted. MADS-box DNA sequences belonging to a homeotic gene family controlling floral development in higher plants are reported for the first time in the genus *Pinus* in this study.

The MADS box gene *AGAMOUS* from *Arabidopsis thaliana* was used as a probe to hybridise with genomic DNA of *P. radiata.* The tentative evidence of hybridisations was obtained in Southern blots, suggesting the possible existence of MADS box related DNA sequences in *P. radiata.* PCR technique was subsequently used to clone these sequences from genomic DNA of radiata pine to confirm the result obtained from Southern blot study . PCR with two degenerate primers targeted to highly conserved regions within the MADS- box resulted in the amplification of a 78 bp DNA sequence. These PCR amplified pine DNA sequences were subcloned in M13 and were sequenced by the dideoxy protocol. The analysis of these DNA sequence data and the amino acid sequences deduced from these DNA sequences showed that these DNA sequences can be divided into three groups, probably belonging to three MADS-box genes of *Pinus radiata.* Two DNA sequence groups are most likely to be the conserved regions of pine MADS-box genes, controlling the late steps of "floral" development which are homologous to class C genes determining the identity of male floral parts (stamens) and female parts (carpels) in angiosperms. One DNA sequence group is speculated to be the conserved region of pine MADS-box gene controlling the earlier steps of floral development, analogous to class B genes controlling petal and stamen development in angiosperms.

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# ABBREVIATIONS

| | |
|---|---|
| *AGAMOUS* gene | *AG* |
| Cetyltrimethyl ammonium bromide | CTAB |
| Dithiothreitol | DTT |
| (3-[4,5-Dimethylthiazol-2-yi]-2,5-diphenyltetrazolium bromide) | MTT |
| Dwarf shoot bud | DSB |
| Endoplasmic reticulum | ER |
| Ethanol | ETOH |
| *FLORICAULA* gene | *FLO* |
| Formalin-acetic-alcohol | FAA |
| Glacial acetic acid | HOAC |
| Isoelectric focusing | IEF |
| *LEAFY* gene | *LFY* |
| Long shoot lateral branch bud | LSLB |
| Long shoot terminal bud | LSTB |
| Methanol | MeOH |
| Nicotinamide adenine dinucleotide | NAD |
| N,N,N',N'-tetramethylethylenediamine | TEMED |
| Phenazine methosulfate | PMS |
| Pollen mother cells | PMCs |
| Pollen/male cone bud | PCB |
| Polyacrylamide gel electrophoresis | PAGE |
| Polymerase chain reaction | PCR |
| Rough endoplasmic reticulum | RER |
| Seed/female cone bud | SCB |
| Sodium dodecyl sulphate | SDS |
| Tertial butyl alcohol | TBA |
| Transmission electron microscopy | TEM |
| Trichloroacetic acid | TCA |

# CHAPTER 1.0 INTRODUCTION

There is no accurate record of how or when radiata pine (*Pinus radiata* D.Don) was first introduced into New Zealand. In a review given by Kininmonth and Whitehouse (1991), it was suggested that miners who travelled the world from one gold rush to another in the early years of the 19th century may have introduced the seed from California to Australia, and then to New Zealand. It was also speculated that the first seedlings were shipped out from England (where radiata pine had been grown since the early 1830s) by some wealthy settler who wished to create park-like surroundings in his new environment. For whatever the reason, Weston (1957) suggested that by 1865, a number of importations of both seedlings and seed, mainly from Australia, had ensured that *Pinus radiata* was well established in New Zealand.

Because of its ease of propagation, rapid height growth, high volume production, and adaptability to a wide range of sites, the Royal Commission on Forestry of 1913 recommended *Pinus radiata* as the most suitable of the introduced tree species for extensive planting in New Zealand.

With the growing interest in this species, it was soon found out that *Pinus radiata* also has a unique wood quality. It has an even-textured, medium-density wood, which is easy to saw, to dry, to treat with preservatives, to machine, to nail, to glue, to stain and to finish. Radiata pine has been proved equally suitable for both interior and exterior use, in structural or non-structural applications. It has also proved very suitable for the manufacture of plywood, particle board and fibreboard, and it provides first-class material for both chemical and mechanical pulping (Kininmonth and Whitehouse, 1991).

Realising its potential significant contribution to the New Zealand economy, a plantation forestry strategy was initiated in the early 1920s. The type of radiata pine typical of New Zealand plantations was derived mainly from Año Nuevo Point and Monterey, Northern California, USA. These natural stands of radiata pine are the ones most likely to be suited to the New Zealand climate out of their five distinct native populations, situated on the coast of California and on two islands west of Baja California, USA.

There have been two periods of intensive plantation development in New Zealand. The first one was in the 1920s and early 1930s, and these plantations are now almost

exhausted and the stands have been re-established. The second one was from the early 1960s until the present day. The net result of this intensive forest plantation is that there is now a total plantation estate of almost 1.3 million hectares, more than 1.1 million (89%) hectares of which is radiata pine. It has been planted throughout most of the 34 to 47$^0$S latitude range in New Zealand (Ministry of Forestry, 1990). Forestry products accounted for 13.5% of New Zealand's total export earnings (NZ$2.6 billion) in the year to March 1994 (Walter and Smith, 1995). This figure is predicted to increase to perhaps 30% by the year 2010 (Ministry of Forestry, 1990).

The successful plantation of radiata pine in New Zealand is not only a result of a climate which benefits tree growth, but also a result of innovative management and tree improvement practices. *Pinus radiata* has been incorporated in breeding projects for more than 40 years. Approximately 400,000 hectares of the radiata pine estate is now established with genetically improved trees (Walter and Smith, 1995). The main type of planting stock used today is seedlings, a small portion of radiata pine stock is from cutting material taken from trees less than five years old (Forest Research Institute, 1990). Breeding projects have been concentrated on the production of seedlots that meet the specific commercial requirements, such as growth rate, log straightness, freedom from major defects, branching habit, wood density and disease resistance.

Carson (1986) reviewed major methods of multiplication of *P.radiata*. He stated that conventional open-pollinated seed orchards established from the mid-1950s to the present time had been successful in meeting total seed demand, and in greatly improving the genetic quality of production stands. He also pointed out that this approach had been found to constrain the potential genetic quality of improved seed by pollen contamination from unimproved trees outside the orchard. Controlled-pollinated seed orchards have been developed as an alternative to the conventional open-pollinated orchards (Sweet and Krugman, 1977). The major advantage of this method is that the pollen contamination can be eliminated. But Carson (1986) pointed out that the cost of seed production from the controlled-pollinated seed orchards is about six times higher than the conventional open-pollinated seed orchards.

Vegetative and micropropagation techniques offered another alternative method for multiplication of improved seed stock, based on a combination of embryo culture and mass culture of shoots arising from cotyledon tissue (Smith *et al*, 1982). Using this

technique, Tasman Forestry Ltd. (a subsidiary of Fletcher Challenge Corporation) had produced between two and three million micropropagated radiata pine *per annum* since 1990. However, field tests of micropropagated material have indicated that growth rates may be less than for seedling stock of similar genetic quality (Smith, 1986).

Clonal forestry, as a promising strategy in the tree breeding programme, has also been critically reviewed by Carson (1986). Clonal forestry was defined as "the establishment of plantations using tested clones". It shares advantages with controlled-pollinated seed orchard strategies, of shorter plant production times, control of pedigree, flexibility of deployment, multiplication of valuable crosses, and efficient capture of additive genetic gains. But it is uncertain whether the genetic gains per unit time, per unit cost, of a clonal forestry system will exceed those achievable by controlled-pollinated seed orchards with or without vegetative multiplication (Shelbourne *et al*, 1989).

With the impact of somatic embryogenesis, Shelbourne *et al* (1989) predicted that it is possible that all steps in clonal forestry from initial multiplication, clone maintenance and commercial multiplication could be done by embryogenesis, and it may provide the impetus needed to make a clonal forestry programme commercially attractive. Furthermore, it could also provide a route towards rejuvenation and to genetic engineering.

In response to this new challenge, the New Zealand Forest Research Institute has been researching embryogenic tissue culture methods for *Pinus radiata* and other conifers for several years. The group, headed by Dale Smith, has been successful in developing reliable methods to regenerate plants by somatic embryogenesis from all clonal families tested. Protocols for somatic embryogenesis of *Pinus radiata* developed at NZ FRI have demonstrated the production of "seedlings" capable of normal growth under forest conditions (Walter and Smith, 1995). Embryogenic tissue of *P.radiata* as a source for genetic engineering is also underway. A molecular biology programme which is integrated into NZ FRI's tree breeding strategy was established in 1992, concentrating on mapping for superior traits, genetic fingerprinting, the development of genetic transformation protocols, and gene expression in conifers. The transformation system of *Pinus radiata* was developed to introduce novel traits into clonal material, with the ultimate aim of generating transgenic trees resistant to herbicides, insects, or other pathogens, and for introducing other desired traits. A protocol to study the transient

expression of a *gus* reporter gene in embryogenic *Pinus radiata* tissue was developed by Walter *et al*, (1994). This protocol was subsequently used to demonstrate stable expression in embryogenic tissue of *Pinus radiata*. Work is now proceeding to select transformed tissue and regenerate plants of this economically important species (Walter and Smith, 1995).

It has to be emphasised that incorporation of transgenic *Pinus radiata* into operational forestry programmes requires many steps before commercial use is possible. Apart from the continuing need to develop efficient gene-transfer methods for commercially desirable genotypes, Strauss *et al* (1995) pointed out that the major constraints to use of engineered trees are ecological safety and regulatory approval. This ecological concern is caused by the movement of transgenes into the environment through the release of pollen and seeds each season, particularly through the release of the massive amount of pollen grains shed in early spring.

It is clear that for both conventional breeding methods and new techniques introduced into the breeding programme of *Pinus radiata*, pollen has always played an important role. Not only that, to plant-based industries dependent on maximal development of vegetative structure such as forestry, nutrient allocation from vegetative growth to reproductive development, such as pollen development has been considered as a waste situation. Pine pollen grains has also been reported as an allergenic source.

Research directed to a greater understanding of the evoking of the male "floral" response and subsequent developmental processes resulting in pollen production in *Pinus radiata* is of vital importance for a good understanding of the processes. Based on this knowledge, manipulative treatments designed to influence the reproductive activity of *Pinus radiata* can be attempted.

It is exactly these goals that set up the targets of this PhD research project. Chapter two of this thesis reviews gymnosperm floral development, especially in relation to the genus *Pinus* and to *Pinus radiata* in particular, and describes a systematic analysis of the morphological and anatomical development of the male cone in *Pinus radiata.* The timing and changes of the structural/ultrastructral features during male cone development were recorded, special attention was given to the timing and cellular changes during the meiotic process, using light and transmission electron microscopy.

In order to accurately detect the ploidy changes during the differentiation of the sporogenous tissue, a rapid and sensitive technique, flow cytometry has been applied by M.E. Hopping (Cytometry Services , Waikanae, New Zealand), in collaboration with this current project. The results of these experiments are presented in **appendix 1**. The results obtained are somewhat controversial and thus are reported as a preliminary study requiring further detailed investigation.

Chapter three of this thesis reports a study on changes of patterns of the total soluble protein by SDS-PAGE and a study on changes of patterns of four isoenzyme systems by isoelectric focusing, aiming at linking the stages of morphological and anatomical development to biochemical markers which represent the new patterns of gene expression which initiate and accompany these changes during male cone development in *Pinus radiata*.

Chapter four of this thesis reviews recent progress in understanding of the genetic control and genetic manipulation of flower development and reports a search for *Pinus* homologues to genes that control floral development in angiosperms.

# CHAPTER 2.0 MORPHOLOGICAL AND ANATOMICAL STUDIES OF MALE CONE DEVELOPMENT IN *Pinus radiata*

## 2. 1. LITERATURE REVIEW

A considerable literature on the phenology of *Pinus* has accumulated since late last century. Species studied include *P. pumilio* (Strasberger, 1872 and 1879), *P. strobus, P. rigida* and *P. austriaca* (Ferguson, 1904), *P. sylvestris* (Haydon, 1907), and *P. laricio* (Coulter and Chamberlain,C.J., 1910). Ferguson (1904) described in detail the sporogenesis of several pine species. A general review of male and female cone development in conifers was presented by C.S. Chamberlain (1935). He described the initiation of microsporophylls and megasporophylls in some conifer species. Doak (1935) and Little (1938) each presented a review on male cone and female cone development in *Pinus*. These early investigators who studied and described the growth and anatomy of male and female cones of pines were mainly concerned with the evolution and morphology of these structures. They attempted to interpret the morphology of bisporangiate cones in the light of angiosperm flowers and the spore-bearing structures of the ferns, and also to identify sequential steps in the evolution of reproductive structures in conifers. Doak (1935) made a comprehensive review of the literature on this subject, and on the basis of a study of some thirty five species of pines, he reinterpreted the previous evidence and added significantly to the knowledge of the evolution and ontogeny of the axial and foliar systems in *Pinus*. He concluded that the male cone axis was homologous to the vegetative dwarf shoot, and the seed cone axis was homologous to the vegetative long shoot. His view indicated that microsporophylls were homologous to the vegetative needle fascicles and megasporophylls were homologous to the vegetative branches. This theory was probably influenced by Goethe's treatise on metamorphosis in angiosperm species (Goethe, 1790). Goethe stated, "Flowers which develop from lateral buds are to be regarded as entire plants, which are set in the mother plant, as the mother plant is set in the earth", indicating that a flower and a shoot might be fundamentally equivalent (Goethe, 1790).

With the introduction of modern microscopy techniques, more species and more thorough investigation of stages in the life history of *Pinus* have been completed. In his book " *The*

*Genus Pinus*", Mirov (1967) compared his own study on *P. edulis* and *P. ponderosa* with that of other researchers' work on *P. roxburghii* (Konar, 1960), *P. halepensis* and *P. pinea* (Francini, 1958), and *P. elliottii* (Mergen and Koerting, 1957). Mirov also presented a complete and extensive review on the general morphological and reproductive growth pattern in *Pinus*.

Electron microscopy has allowed detailed information on microsporogenesis, megasporogenesis and embryogenesis in *Pinus* to become available. This is reviewed in the book "*Embryology of Gymnosperms*" (Singh, 1978). So far, most of the information has been obtained from studies of *Pinus* growing in the northern temperate zones. The latest general review of these studies has been presented by Owens (1985). In his review, he stated that reproductive buds of pines underwent early development before winter dormancy and overwintered at various stages. Pollination occurred in the spring or early summer of the second year, pollen tubes and ovules partially developed but then stopped, usually in mid-summer. Development resumed the following spring; fertilization occurred and seeds were mature in autumn. Seeds were usually shed in the year they matured. The reproductive cycle took a minimum of three years, with commonly about 27 months from reproductive bud initiation to seed maturity. He reported that the time of the reproductive bud initiation in the life of pines and during the growing season might vary from one species to another as might the sites of cone buds in the crown and on the shoot. He also reported that seed-cone buds were initiated in a complex vegetative long-shoot bud rather than on an elongating shoot or on a dwarf shoot. The long shoot bud consisted of a series of scale leaves (cataphylls) which were initiated throughout the growing season. Most cataphylls had an axillary apex which initiated a series of bud scales, then differentiated into a dwarf shoot, pollen-cone or lateral long shoot bud. The time when an axillary bud differentiated was determined by its position in the long shoot bud--the proximal buds which were initiated first differentiated before the more distal axillary buds.

This review was based on studies on *Pinus* by a number of researchers, including study on the evolution of foliar types, dwarf shoots, and cone scales of *Pinus* by Doak (1935), study on the structure and seasonal activity of the shoot apices of *P. lambertiana* and *P. ponderosa* (Sacher, 1954), study on the time scale of morphogenesis at the stem apex of *P. resinosa Ait* (Duff and Nolan, 1958), study on the shoot apex in eastern white pine *(P. stribus)* (Owston, 1969), study on the bud development in lodgepole pine (*P. contorta)*

(Van den Berg and Lanner, 1971), study on the timing and rate of bud formation in *P. resinosa* (Sucoff, 1971), study on the developmental anatomy of long-branch terminal buds of *P. banksiana* (Curtis and Popham, 1972), study on the vegetative buds and shoots of lodgepole pine (*P. contorta)* (Lanner and Van den Berg, 1975), and study of the development of long-shoot terminal buds of *P. contorta* and *P. monticola* (Owens and Molder, 1975, 1977). It has also been reported that the growth of long-shoot buds could be monocyclic consisting of one complete sequence, or polycyclic consisting of two or more sequences(Owens, 1985). In general, complex polycyclic growth was characteristic of warm temperature or tropical climates, as observed in young caribbean pine (*P. caribaea var. hondurensis)* (Chudnoff and Geary, 1973) and *Pinus radiata D.Don,* growing in New Zealand (Bollman and Sweet, 1976).

More detailed descriptions of the initiation of the male cones of the genus *Pinus* and their subsequent development have been given by a number of researchers. Owens and Molder (1976) observed the initiation of the male-cone buds of western white pine (*Pinus monticola*). They found that pollen-cone bearing terminal buds differentiated from proximal axillary primordia on smaller, less dominant, lateral branches in lower regions of the crown. About two thirds of the total number of axillary primordia differentiated into pollen cone buds. After an average of 17 sterile cataphylls were produced at the base of the axillary primordia, the axillary buds began to acropetally differentiate into pollen cone buds. They found that the potential pollen cone apices were larger than similarly positioned potential dwarf shoot apices, and microsporophylls were initiated as rounded buttresses, which elongated perpendicular to the cone axis to form truncated primordia. There was a decrease in length acropetally in each pollen-cone bud. Even after all the microsporophylls were initiated , the sporogenous tissue did not form before the period of dormancy of the cone bud in November. Mergen and Koerting (1957) observed the development of the microsporophylls of the male-cone buds in slash pine (*Pinus elliottii*), growing in the south-eastern part of the United States of America. They found that during the latter part of July the lower meristematic area of the male-cone primordia started to differentiate hood scales which began to envelop the rudimentary strobilus and by September 13, up to eight layers of scales had formed. The innermost layer pushed between the primordia and the previously formed scales curved inward near the apex, and formed a protective arch. They found that these scales had a thick epidermis, especially in

the outer surface, which became suberized during early development. At this stage the strobili primordia had not formed a protective cuticle and the authors suggested that this hood might prevent excessive evaporation from the succulent structure. During the latter part of September the axis of the male-cone started increasing its length and the rudimentary microsporophylls started to differentiate at the base. By October 4, sporogenous initials had been laid down in the abaxial part of some of the early microsporophylls.

Detailed descriptions of the microsporogenesis of other pine species are also available. Generally in *Pinus*, the sporangia initials lie on the surface of the sporangium-forming meristem in the abaxial part of the early microsporophyll. Following periclinal divisions in the initial cells, the outer layer of cells divides only anticlinally (these cells could thus be labelled as epidermis) and the inner cells (primary archesporial cells) divide in all planes to produce a mass of archesporial cells. The peripheral cells of this mass divide periclinally to produce a subepidermal primary parietal layer which forms the wall layers. The innermost layer of the wall has been called a "tapetum", by analogy to the angiosperm tapetum. All of these wall layers develop from the vegetative tissues of the microsporophyll. The archesporium gives rise to the sporogenous cells which differentiate into pollen mother cells (PMCs). Konar's research in *Pinus wallichiana* (Konar, 1957) found that the epidermis of the microsporangium consisted of broadly elongated or isodiametric cells which underwent frequent anticlinal divisions. Their outer wall was cutinized and most of them were filled with a uniformly staining tannin-like material which persisted until the time of pollen shedding. Frequently the subepidermal layer was also filled with a homogeneously staining substance. The cells of the third layer became tangentially elongated and were the first to degenerate. According to its orientation and the size of the cells, Konar (1957) suggested that the innermost tapetum appeared to be a derivative of the parietal layer, the cell layers outside the innermost tapetal layer. Singh (1978) clearly confirmed that the tapetum rose from the innermost wall layer in the dorsal portion of the sporangium and contiguous vegetative cells in the ventral portion, not from outside cell layers.

He also gave a detailed review of the cellular changes of the tapetum during the differentiation of the pollen mother cells. He stated that the tapetum comprised a single (occasionally two ) layer of large, richly cytoplasmic and multinucleate cells encircling the

sporogenous tissue in the early stage of the microsporangium. The cells were connected to each other by broad cytoplasmic channels in *Pinus*. The syncytium character of the tapetum cells was supported by the work of Dickinson and Bell (1976a) with *P. banksiana*; they found that cytoplasmic organelles like mitochondria could even pass through these channels. Based on a number of researchers' studies, Singh (1978) reviewed the general pattern of tapetal development. The tapetum cells showed their best development during meiosis in PMCs and usually degenerated soon after microspores were released from the tetrad. There appeared to be a correlation between the stage of the meiocyte and the structure of tapetal cytoplasm and three developmental phases were distinguished. Early in their development the tapetal and the sporogenous cells were ultra-structurally alike with rich cytoplasm and a multinucleate appearance. Soon afterwards, the protoplasm of the tapetal cells began to shrink and small quantities of a fibrous material accumulated between the protoplasm and the cell wall. From this stage onward, the tapetal and the sporogenous cells became distinct from each other. Large accumulations of rough endoplasmic reticulum and other associated coated vesicles became evident in the tapetal cells . Mitochondria increased in size and frequency and the ribosomal population rose very sharply. Shortly before the pollen mother cells entered prophase of meiosis the rough ER became conspicuously layered at the periphery of the cytoplasm. The volume of tapetal cytoplasm increased sharply, and numerous golgi bodies were present in the tapetal cytoplasm during the zygotene stage. The tapetal cells also started producing small vesicles during the pachytene-diakinesis stage of meiosis of pollen mother cells. As the walls of the tapetal cells became gelatinous, an electron-dense globular material was found on the outer tangential middle lamella. Also, unnamed granules, comprising the same kind of material as the lipid layer of the tapetal membrane, made their appearance among the fibrillar matrix of the degenerating tapetal cell walls. The number and size of these unnamed granules increased during interphase. The electron-dense globular material which had occurred during diplotene and interphase in microspore mother cells disappeared completely at a later stage. It has been reported that this electron-dense globular material might contain sporopollenin, but in a different state from that around the unnamed granules or on the tapetal membrane (Dickinson and Bell 1976b, Vasil and Aldrich 1970, Willemse 1971).

For the development of the sporogenous tissue in *Pinus,* Dickinson and Bell (1976b) reported that the differentiation of the sporogenous tissue starts in the centre of the sporangium and proceeds centrifugally. They pointed out that it was because of the absence of protoplasmic connections between the sporogenous cells. The implication here is that if protoplasmic connections had been present, then the development would have proceeded simultaneously. In her thorough investigation of pine life histories at the beginning of this century, Ferguson (1904) also recorded the cell structural changes during the differentiation of the sporogenous tissue. She found that the cells of the primitive archesporial cells were larger, and that they had larger nuclei and denser cytoplasm than those of the wall layer cells . They were also polyhedral in outline. The nucleus of the archesporial cell contained several nucleolus-like bodies; as many as 11 were counted in a single section. She reported that during the period preceding the reduction division, the archesporial cells differentiated into pollen mother cells. They increased their size so that the nucleus of PMCs became even larger than an entire cell of the original archesporium. The walls of the PMCs thickened considerably, and the cytoplasm assumed a fine, almost granular structure which under high magnification, resolved itself into a delicate close reticulum. She also noticed that as the nucleus of a pollen mother cell enlarged, its reticulum became more open, the threads of the net gradually increased in thickness. As soon as PMCs attained their full size, the prophase of the first division was initiated. The reticulum gradually drew together, its threads became thicker and the meshes became smaller. Contractions of chromosomes continued until the network formed a compact mass at one side of the nucleus. During synapsis the nucleoli was entirely confined within the contracted sphere. PMCs continued reduction division events which yielded haploid pollen grain as a result (Ferguson 1904).

Konar (1957) also observed the process of meiosis in *P.wallichiana.* He found that the premeiosis PMCs were polygonal in shape, each with vacuolate cytoplasm and a prominent nucleus almost filling the entire cell. During meiosis, the cytoplasm of the PMCs rounded up and a special mucilaginous wall was secreted, the middle lamella dissolved and the cells were separated from each other. Wall formation in PMCs in *P.wallichiana* was simultaneous and callose walls between the four nuclei grew centripetally, like most gymnosperms reported by Singh (1978). After the young microspore emerged from the original wall and separated from each other, the pollen grain

soon developed two extensions of the exine which formed the two air sacs or bladders on each side. These bladders were formed between intine and exine of the pollen wall. The microspore nucleus generally lay towards the distal end and divided to form two cells. The small lenticular cell cut off was the first prothallial cell. This soon degenerated. The next divisions cut off the second prothallial cell which was similar to or at times slightly smaller than the first. By the time the third mitosis occurred in the central cell nucleus, the second prothallial cell had also degenerated and the two were merely seen as dark streaks at the distal end of the pollen grain. The third division resulted in the formation of a generative cell and an ovoid tube nucleus. The tube nucleus was always larger and contained looser chromatin. The pollen was shed at the four-celled stage (Kornar, 1957,1960). Mergen and Koerting (1957) reported that from the start of the reduction division to the second vegetative division of the microspore in slash pine (*P. elliottii*) the development was very rapid. If weather conditions were favourable, all stages from the microspore-mother-cell stage, through the tetrad stage, to the second vegetative division of the microspore were seen among the pollen cone buds collected over a three day period from the same tree.

*Pinus radiata*, (radiata or monterey pine) has been well established since its introduction into New Zealand from California, USA in 1865 (Weston, 1957). As the climate is much warmer throughout the year, New Zealand offers a much more favourable and longer growing season. *Pinus radiata* growing in New Zealand shows some unique features during its life cycle, which differ greatly from other pine species growing in the northern temperate-zone. The broad picture of the growth pattern in *Pinus radiata*, the timing of long shoot initiation of leader and branch shoot, and the morphology of the long shoot development is already known (Bollmann and Sweet, 1976, 1979; Bollmann, 1983). A polycyclic growth pattern of the long shoot has been confirmed (Sweet and Bollmann, 1976). The initiation of the components of the annual shoot begins between mid-September and mid-October and finishes during August in Rotorua, in the central North Island of New Zealand. Five clusters of branches develop each year, the first three of which bear seed cones. These three branches are initiated in December, at the end of January, and during March. The appearance of the sterile cataphylls represent the start of a growing cycle of the long shoot, while a cluster of branches and/or seed cones represent

the end of a growing cycle. After a number of cataphylls are laid down by the apical meristem of the leading and branch shoot, axillary primordia develop in the axis of cataphyll below the forth sterile cataphyll from the apex. Soon after initiation, axillary primordia start to form their own axillary bud cataphylls. Axillary primordia could be short-shoot or long-shoot primordia; short-shoot primordia occur within a cycle, and long-shoot primordia occur at the end of a cycle. Short-shoot primordia, normally developing into needle fascicles might be modified to form pollen cones; long-shoot primordia might develop into branches or be modified to form seed cones (Bollmann and Sweet, 1976, 1979; Bollmann, 1983).

Apart from these extensive studies on the general growth pattern of the radiata pine, a detailed study of the seed cone developmental process from the seed cone initiation to seed maturity was also completed to meet the needs of large breeding programme for *P. radiata* in New Zealand (Lill 1975,1976; Bollmann and Sweet 1976). These workers found that the development of ovule tissues in *Pinus radiata* after meiosis, fertilization, and embrogeny was comparable with that of other pines, but *P.radiata* took longer to develop. Fertilization occurred 15 months after pollination and morphological embryo maturity was reached five months later (Lill 1975, 1976; Bollmann and Sweet 1976). However, there is little published information on male cone developmental processes from cone initiation to pollen maturity in *Pinus radiata.*

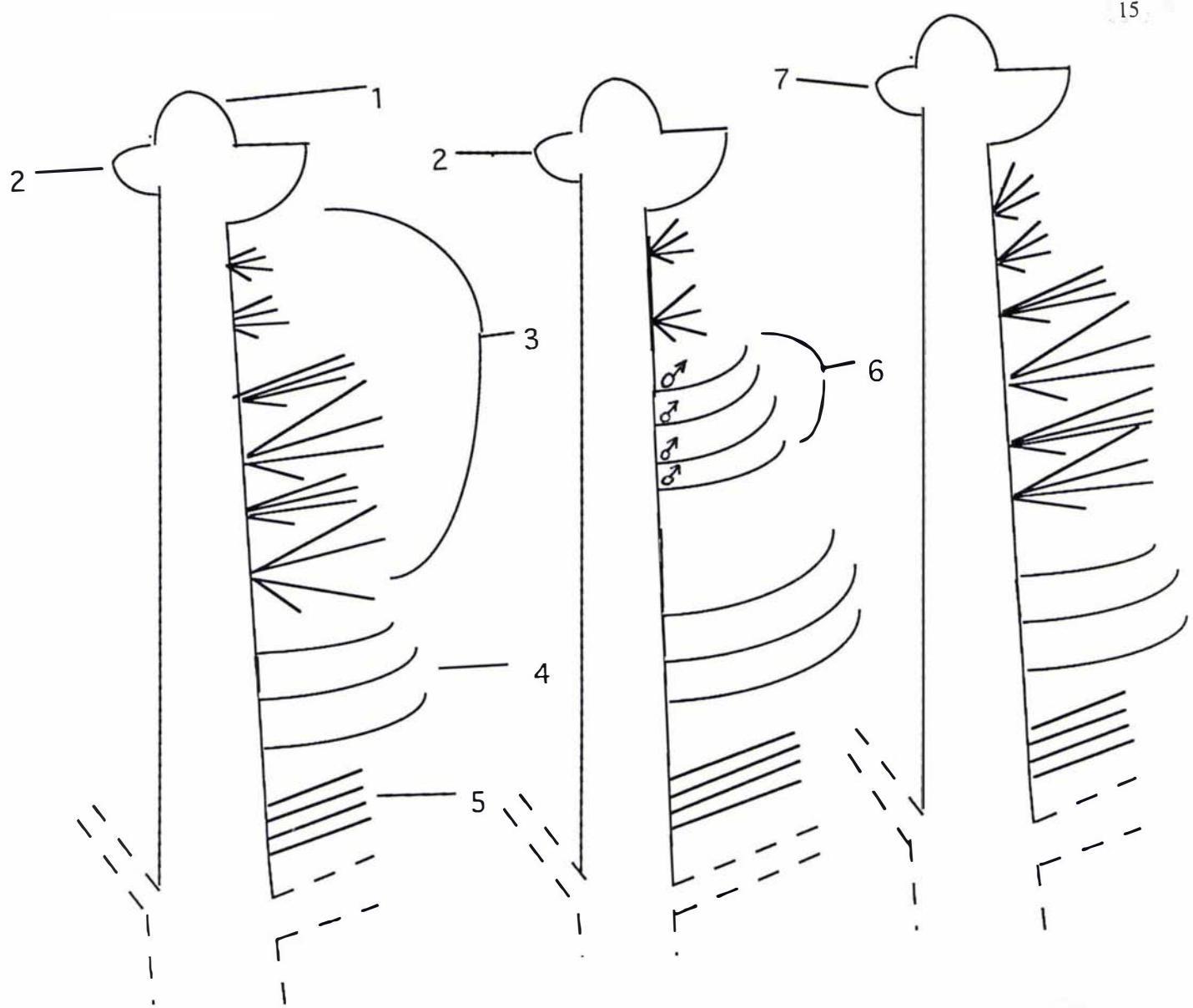
This study presents the result of a microscopy study which was designed to determine the time of the initiation of the male cone and the subsequent development of the male floral structures on *Pinus radiata* clonal trees growing in the Rotorua area. In an extensive light microscopy study, tissue and cellular changes from the initiation of the male cone to the formation of mature pollen are described. Selected stages within this development process were explored further at the cell and subcellular levels, using electron microscopy to further describe the nature and the timing of the developmental events. In particular, the nature of the connection and disconnection among pollen mother cells and tapetal cells, the fate of tapetal cells, and the reorganisation of the cellular organelles of pollen mother cells and tapetal cells during the presumptive meiotic processes were examined.

The present study reports on the male cone development in *Pinus radiata* using the terminology of Owens and Molder (1975, 1977). In northern temperate pine, lateral

branches are terminated by a long shoot terminal bud (LSTB). Each year the apical meristem of this LSTB forms a complete preformed, telescoped shoot axis, which overwinters and elongates the following spring. The composition of the shoot axis is thus determined by the organogenic sequence of the apical meristem, which begins with the initiation of sterile cataphylls as the subtending preformed shoot axis begins to elongate. The initiation of sterile cataphylls is then replaced by the initiation of fertile cataphylls bearing axillary apices. These apices are then anatomically differentiated with the formation of leaf primordia, fertile cataphylls, microsporophylls, or bracts corresponding to the differentiation of vegetative dwarf shoot buds (DSB), long shoot lateral branch buds (LSLB), male/pollen cone buds (PCB), or seed cone buds (SCB). Most of the axillary buds develop as DSB or PCB, but a few of the more distal axillary buds develop as LSLB or SCB. The completion of an organogenic sequence is signalled by the resumption of sterile cataphyll initiation. The terminology developed by Owens and Molder is shown diagrammatically in Fig 2.1 where it is used in the context of reporting results.

Fig 2.1. (upper) Diagrammatic representation of one cycle of shoot development of three types of long shoot terminal buds (LSTB) in *Pinus radiata* (solid lines).
1: Terminal bud, 2: Lateral bud to form a branch, 3: Needle fascicle,
4: Widely spaced sterile cataphylls, 5: Closely spaced sterile cataphylls
6: Male cones, 7: Lateral bud to form a seed cone.
Adapted from Cremer (1992).

(lower): Subordinate shoots of *Pinus radiata*, showing clusters of mature pollen cones (arrow 1) and the putative vegetative dwarf shoot buds zone above them (arrow 2).




Vegetative LSTB

Male LSTB

Female LSTB

# 2. 2. MATERIALS AND METHODS

## 2.2.1 SAMPLING OF THE MATERIALS

Pollen cone-bearing shoot terminal buds were collected from three 30-year-old *Pinus radiata* clonal trees, 880-606, 880-607, and 880-612, growing in the New Zealand Forest Research Institute nursery in the central North Island, Rotorua, New Zealand (latitude $38^0$24' and altitude 544 m). The annual mean average daily temperature is $11^0$C; mean winter temperature is $7.46^0$C and mean summer temperature is $16.57^0$C (Bollmann and Sweet, 1976). Collections were made weekly or fortnightly from early November 1991 to late July 1992. On each collection date, branches which bore pollen cones were sampled from the middle of the crown. For the result reported in this chapter, only clone 880-606 was used. Before mid-April, the male long shoot terminal buds were fixed in formalin-acetic-alcohol (FAA) for light microscopy study. After mid-April, only the male cone buds collected from the middle region of the pollen cone-bearing shoot were fixed for examination by this method in this light microscopy study. Microsprophylls removed from the middle region of male long shoot terminal buds collected on 19/4, 20/5, 27/5, 3/6 and 16/6, 1992 were fixed in modified Karnovsky fixation fluid (1965) for transmission electron microscopy study. To investigate variations in the size of male cone buds along a shoot during the growing season, male cone buds collected on 27/5, 3/6, and 2/7, 1992 were measured. From the bottom to the top, the length and the maximum width of each male cone bud on the selected male cone bearing shoot from these three stages were measured.

## 2.2.2. METHODS IN LIGHT MICROSCOPY

2.2.2.1. Fixation

Samples were fixed in Formalin-acetic-alcohol (FAA).

Formalin-acetic-alcohol (FAA)

| | |
|---|---|
| 70% ethanol alcohol | 450 ml |
| Glacial acetic acid | 25 ml |
| Formalin | 25 ml |

2.2.2.2. Dehydration

Samples were dehydrated using Johansen's ethyl alcohol/tertial butyl alcohol (TBA) method (Johansen, 1940).

Procedure:

Samples from the fixation fluid were transferred sequentially through an alcohol series (10%, 20%, 30%, 50% 70% ethyl alcohol) allowing 30 minutes in each, then transferred to 95%, 100%, alcohols and 75% absolute alcohol + 25% TBA for 2.5-3 hours each. Samples were then transferred to pure TBA with three changes over 24-36 hours.

2.2.2.3. Infiltration with paraffin

Material was transferred to TBA/paraffin (50:50) and held at $56^0$C for 1 hour, then transferred onto solid paraffin in the prepared vial. The vial was placed in the oven at $60^0$C. The melted paraffin oil covering the material protects it from heat damage, and as the solid paraffin melts, the material will sink and become progressively infiltrated with paraffin. When the paraffin is completely molten, the material was transferred to fresh paraffin. This transfer was repeated three times over a period of 24 hours, to remove all traces of TBA.

2.2.2.4. Embedding

The material was embedded in paraffin (melting point $56^0$C) in suitable sized porcelain "boats", which were then placed on a cooling plate, to allow even solidification.

2.2.2.5. Sectioning

The material was sectioned with a rotary microtome (REICHERT, Austria) set at 8 μm.

2.2.2.6. Mounting the sections

Sections were mounted on microscope slides using 10% P.V.A.White Resin Glue (National Starch & Chemical NZ Ltd).

2.2.2.7. Staining

Sections were double stained in alum-haematoxylin and safranin, according to the staining protocol developed in the laboratory of J.N. Owens, University of Victoria, Canada (personal communication).

2.2.2.8. Staining procedure

Xylene 2-5 minutes
100% alcohol 2-5 minutes
95% alcohol 2-5 minutes
70% alcohol 2-5 minutes
50% alcohol 2-5 minutes
30% alcohol 2-5 minutes
Distilled $H_2O$ 1-2 minutes
Safranine (0.5% in 60% alcohol) 60 minutes
4 times change in distilled $H_2O$
4% mordant iron alum 30 minutes
4 times change in distilled $H_2O$
0.5% aqueous haematoxylin 60 minutes
3 changes in distilled water ⟶ Destaining in 2% mordant iron alum
3 changes in distilled water
slow running tap water
50% alcohol
70% alcohol
95% alcohol
100% alcohol I
100% alcohol II
Xylene I
Xylene II
Xylene III
D.P.X.mountant (BDH) and cover slide

The mordant solution :

4% iron alum 500 ml
Acetic acid (glacial) 5 ml
10% $H_2SO_4$ 6 ml

Destaining reagent: Dilute the mordant solution with an equal volume of water.

Fresh squashed mature pollen cone tissues were stained with a pollen specific stain.

Pollen stain:

Glycerol 16% v/v
Ethyl alcohol 33% v/v
Basic fuchsin 0.02% w/v

2.2.2.9. Light microscopy examination

Stained sections were examined using a Zeiss bright field microscope and photomicrographs were taken using colour slide film, Epy 160 Tungsten.

## 2.2.3. METHODS IN TRANSMISSION ELECTRON MICROSCOPY

2.2.3.1. Fixation

Microsporophylls collected from male cone buds at the selected stages were fixed in the modified Karnovsky fixation fluid (Karnovsky, 1965).

Procedure for making the Karnovsky fixative:

1. 2 gram paraformaldehyde dissolves in 50 ml distilled water, heat to $65^0$C to dissolve.
2. Drop a pinch of sodium hydroxide (NaOH) to clear the solution, and allow to cool.
3. Filter the solution, and add 12 ml 25% gluteraldehyde.
4. Add 2.51 gram $Na_2HPO_4.12\ H_2O$, 0.41 gram $KH_2PO_4$.
5. Adjust PH 7.2, add distilled $H_2O$ into 100 ml.

Final solution is 2% formaldehyde, 3% gluteraldehyde and 0.1 M $PO_4^{3-}$ PH 7.2

The fixative was washed off in 0.1 M $PO_4^{3-}$ buffer (pH 7.2) for three times, each time allowing 30 minutes.

The samples were transferred into small vials, Thirty drops of 0.1 M $PO_4^{3-}$ buffer (pH 7.2) and ten drops of osmium tetroxide ($OsO_4$) were added to each vial, and the vials were stored in the refrigerator for one hour.

The osmium tetroxide ($OsO_4$) was washed away by three changes of distilled water, each time allowing 5-10 minutes. The samples were left in distilled water at $4^0$C overnight.

2.2.3.2. Dehydration

The samples were dehydrated through an acetone series (20%, 40%, 60%, 80%) allowing a minimum of 20 minutes in each, and three changes in 100% acetone, each time allowing 5-10 minutes.

2.2.3.3. Infiltration

Two third (2/3) of Acetone (absolute) and one third (1/3) of resin (Vinylcyclohexene dioxide VCD-DER 736) (Spurr, 1969) were firstly added into each sample vial, and left

for 8-24 hours, and then replaced by 1/3 of acetone (absolute) and 2/3 of resin, and left for 8-24 hours. At last only pure resin was added into each sample vial.

2.2.3.4. Embedding

The samples were transferred into clean dry capsules. The capsules were filled with fresh embedding resin (Vinylcyclohexene dioxide VCD-DER 736) and transferred into a oven baking at 60°C overnight, to ensure the resin had evenly polymerised.

2.2.3.5. Sectioning

Sections were examined under the light microscope first to locate the particular cells needed to be examined by electron microscopy, the procedure was as follows:

1. The resin block surface was trimmed into about 2 $mm^2$ with a trapezium shape. The resin block was sectioned with a glass knife at the thickness of 1 μm on a Reichert Ultracut Microtome.

Sections was stained with 0.05% toluidine blue dissolved in 0.1 M $PO_4^{3-}$ buffer (pH 7.2) for 2-3 minutes and examined under the light microscope, to locate the particular cells of interest for further examination using electron microscopy.

2. The resin block was then sectioned with Diamond knife on the microtome at the thickness of 0.1 μm. The sections were picked up with 200 or 400 mesh unsupported grid. (a little sellotape chloroform was dropped on the grids before use). The grids were dried on filter paper, and stored under a clean petri dish.

2.2.3.6. Staining

The grids were stained following the methods of Roland and Vian (1991).

1-2% aqueous uranyl acetate (filtered before use) 3 minutes, 50 % alcohol 30 seconds, MilliQ $H_2O$ 10 seconds-1 minutes, and lead nitrate 3 minutes. The grids were rinsed with three changes of MilliQ water in the end.

After the grids had been air dried at room temperature under a clean petri dish for 10-15 minutes, they were ready for electron microscope examination.

2.2.3.7. Transmission electron microscope examination

Sample grids were examined under a Phillips 201C transmission electron microscope, with an accelerating voltage of 60 KV.

# 2. 3. RESULTS

## 2.3.1. MORPHOLOGICAL ASPECTS OF THE MALE CONE DEVELOPMENT IN *Pinus radiata*.

The three types of long shoot terminal buds (LSTB) in *Pinus radiata* were examined in this study. (vegetative LSTB, male LSTB and female LSTB). Vegetative LSTB were composed almost entirely of vegetative dwarf shoot buds (DSB) with a few distal long shoot lateral buds (LSLB) and no reproductive buds. Female LSTB were similar but had seed cone buds (SCB) as well as LSLB in the distal position. Male LSTB had a basal zone of pollen cone buds (PCB), followed by a zone of DSB, and finally a few LSLB. A diagrammatic representation of these three types of LSTB and subordinate shoots bearing clusters of pollen cones is shown in Fig 2.1.

Male LSTB are not leading shoot buds, they form only on first or second order branches, usually in the lower part of the crown. These subordinate shoots are unicyclic producing only one cycle of growth per year and they are predetermined in the sense that the structures expanded in one year were presented in the overwintering buds, having been initiated in the previous growing season.

To investigate the possibility that individual male cones on a shoot axis were undergoing development at different rates, male cone buds from three different stages were removed from the shoot, and their length and maximum width measured (Fig 2.2).

This study shows that the basal pollen cones which were usually 40-50 mm away from the shoot apex were slightly longer compared with the distal pollen cones which were usually located within 20 mm from the shoot apex. But the differences in width between male cones of these two regions were not so significant, and the most basal one was not necessarily the largest pollen cone in size (Fig 2.2).

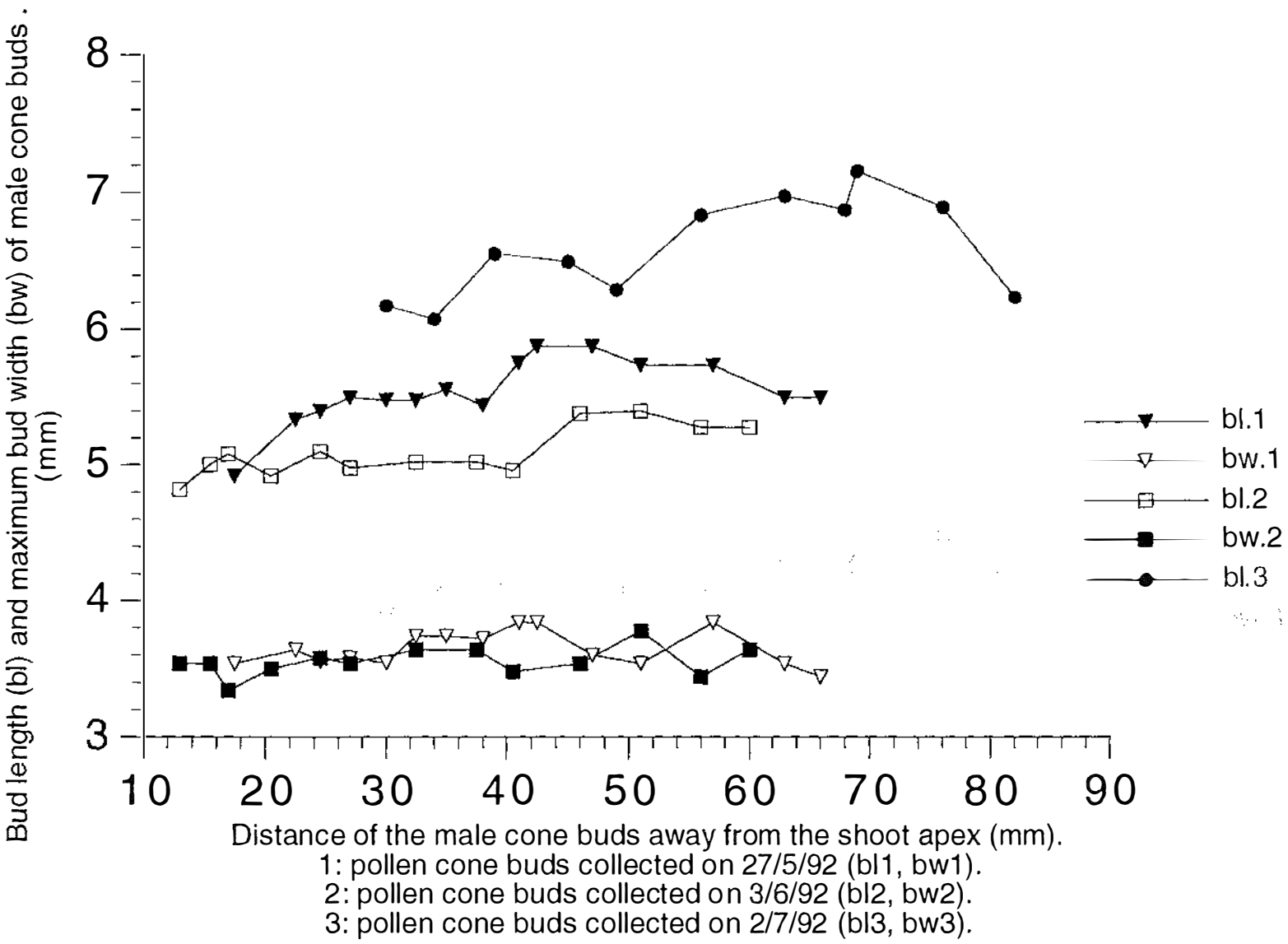



Fig 2.2 Changes of the size of male cone buds at different locations on the shoot axis from three developmental stages.

## 2.3.2. ANATOMICAL STUDY OF THE MALE CONE DEVELOPMENT IN *Pinus radiata* RESULTS FROM LIGHT MICROSCOPY

This study used the Iron-haematoxylin (Heidenhain's) and safranin double staining method, recommended by J.N. Owens' laboratory. This method works very well on most mature tissues, especially on conifers. Hematoxylin stains chromosomes and unlignified walls blue-black to black, and cytoplasm grey. Safranin stains lignified tissue, and suberized and cutinized cell walls red.

The youngest long shoot terminal buds (LSTB) for this study were collected in November 1991, when each LSTB apical meristem was encased in a series of broad, sclerified, achlorophyllous sterile cataphylls (Fig 2.3), formed from pockets of localised mitotic activity within the peripheral zone of the LSTB apical meristem. There were no signs of axillary apices. By December 5, random division of hypodermal cells in the peripheral zone around the apical meristem caused buttresses to develop from the apical flank (arrow 1, Fig 2.4). Epidermal cells divided periclinally and the primordium grew outwards. Gradually a meristem appeared along the primordium margin, and a broad flattened sterile cataphyll was formed (arrow 2, Fig 2.4). A proximal to basal developmental gradient was apparent. The forming of sterile cataphylls signalled the beginning of a single growing cycle of the LSTB. After four to five sterile cataphylls were formed, the fertile cataphylls started to develop in the same manner as sterile cataphylls, except that each bore an axillary apical meristem (arrow 3, Fig 2.4). The axillary apices developed from pockets of mitotically active cells in the axil of the cataphyll. They remained relatively small and took on a faintly stained apical zonation. These axillary apices were also the earliest formed short shoot initials. It was these predetermined buds which eventually developed into pollen cone buds. This determination is based on their location in the shoot apex, but it must be emphasised that the appearance of these axillary primordia at this stage does not necessarily mean that they are male cone primordia; vegetative dwarf shoot buds (DSB) which will develop into needle fascicles have a similar appearance at this stage.

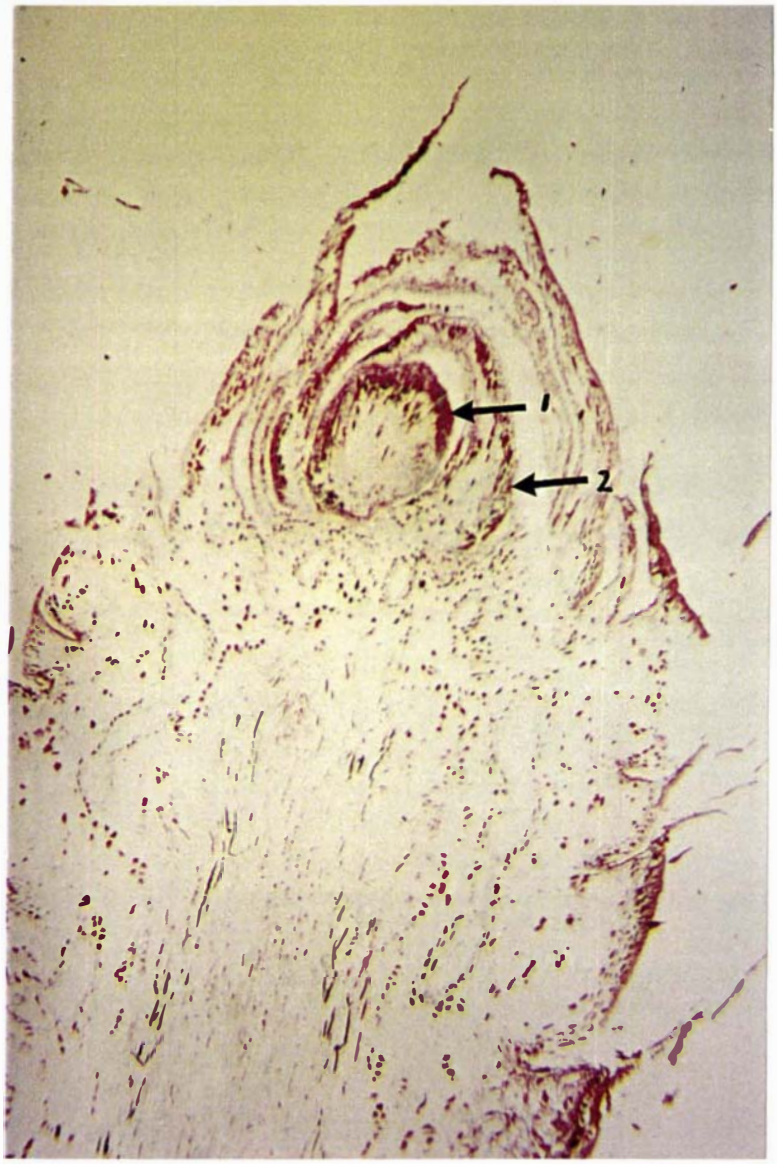



Fig 2.3. A longitudinal section of a subordinate shoot terminal bud collected in mid-November 1991, showing the apical meristem (arrow 1) encased in a series of sclerified sterile cataphylls (arrow 2). x25

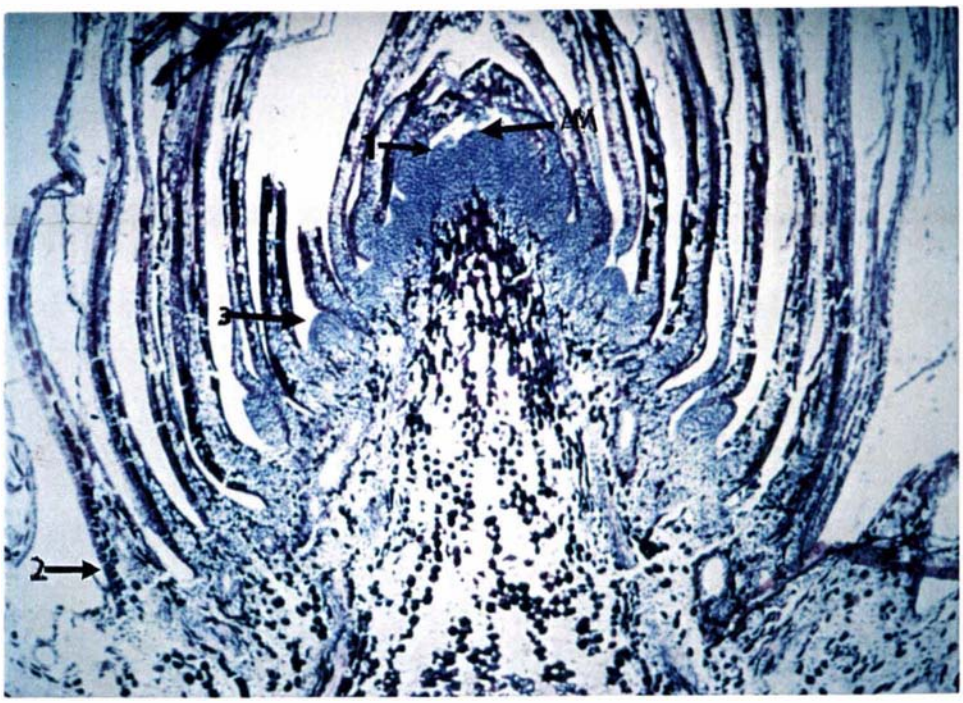



Figure 2.4. A median longitudinal section of a subordinate shoot terminal bud collected in early December 1991, showing the apical meristem (AM), a buttress developed from the apical flank (arrow 1), a sterile cataphyll (arrow 2) and the axillary apical meristem in the axis of a fertile cataphyll (arrow 3). x40

The axillary apices became increasingly larger, as cell division continued, and by January 15 bullet-shaped apices had been formed below the three to four earlier-formed axillary bud cataphylls (arrow 1, Fig 2.5). These bullet-shaped apices were interpreted as potential pollen cone buds (PCB). The upper 3-4 axillary buds were smaller, less developed, and had slightly smaller apices than the lower bullet-shaped pollen cone buds (arrow 2, Fig 2.5). This was the earliest stage when it was possible to identify confidently these axillary structures as differentiating pollen cone buds. Microsporophylls within these buds could not be seen at this stage, but potential microsporophyll initials are shown in Fig 2.6.

Pollen cone buds had begun to differentiate by late February. After the sterile cataphylls were initiated at the base of the potential PCB, the bullet-shaped apices lengthened tall and microsporophyll buttresses were produced by hypodermal cell division in the lower apical flanks ( Fig 2.7). Microsporophyll initiation advanced acropetally, and the apex of the pollen cone bud was gradually diminished with each microsporophyll until the apex finally disappeared ( Fig 2.8). By early March microsporophyll initiation was completed.

By mid April, the differentiation of the microsporophyll had progressed towards the apex and the completely formed microsporophylls were well turned upwards (arrow 1, Fig 2.9). This was probably the result of asymmetrical growth; cell division, both periclinal and anticlinal, was probably more rapid in the abaxial area than the adaxial surface. Sporogenous initials had been laid down in the abaxial part of some of the earliest formed microsporophylls by this time (arrow 2, Fig 2.9), and in the earliest formed microsporophylls these sporogenous initials had already given rise to sporogenous tissue. Within the cone, there was a clear indication of differentiation to sporogenous tissue in the lower earlier formed microsporophylls, while the upper later formed ones were undifferentiated. This difference in maturity within a cone was maintained through to pollen release. For this reason, the developmental stages shown in Figs 2.11 - 2.15 are taken from microsporophylls from the middle region of the male cones.

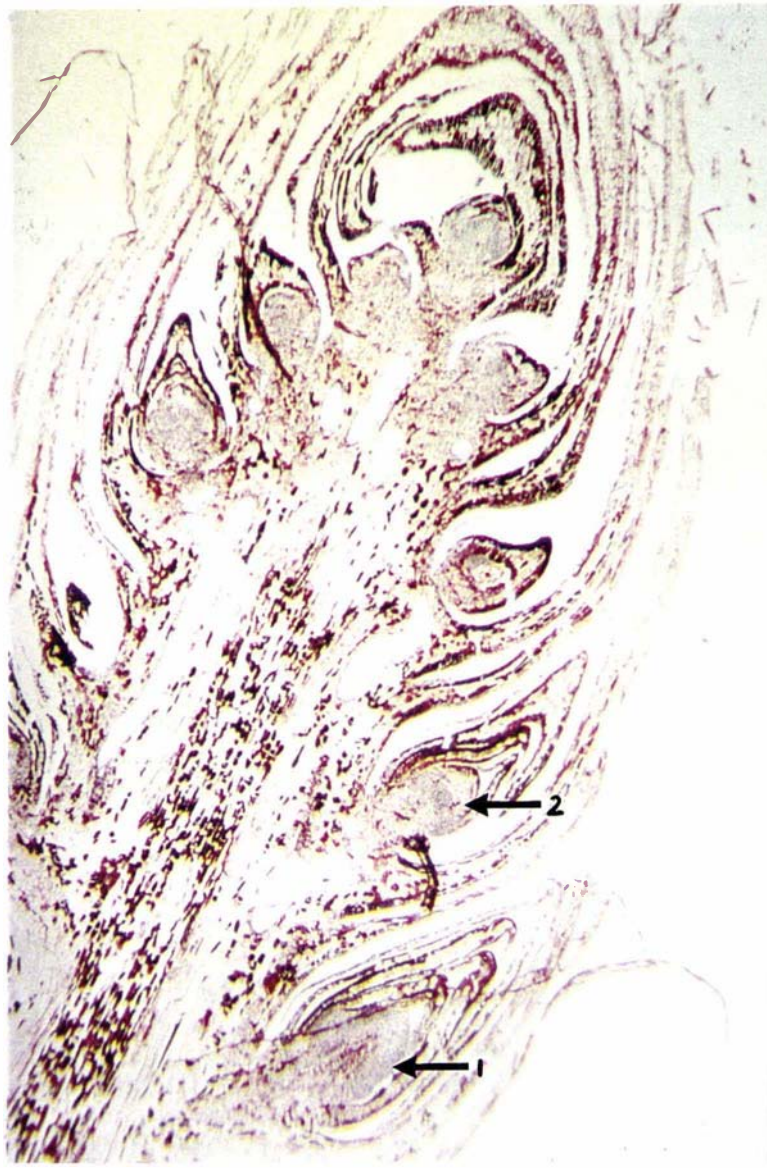



Fig 2.5. A longitudinal section of a subordinate shoot terminal bud collected in mid-January 1992, showing the earliest formed pollen cone bud (arrow 1), and some smaller axillary buds (arrow 2) above the pollen cone bud. X25

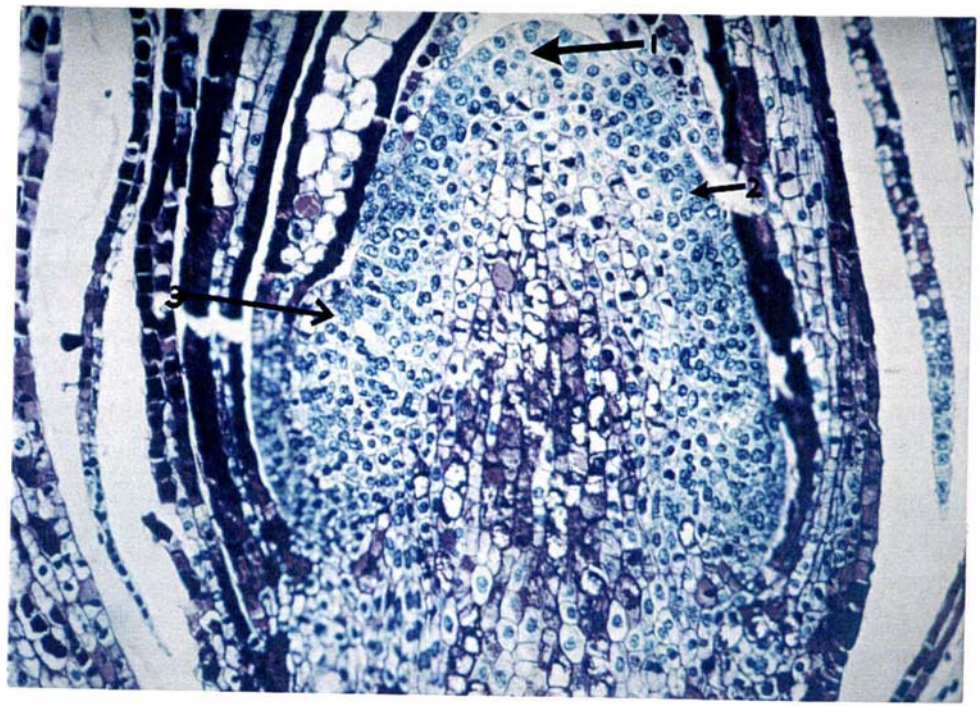



Fig 2.6. A higher magnification of Fig 2.5, showing the relatively larger and bullet-shaped apex (arrow 1), the potential microsporophyll initials, (arrow 2, the strongly stained cell zones), the initiation of the pollen cone bud sterile cataphyll (arrow 3), and the central pith cells. x160

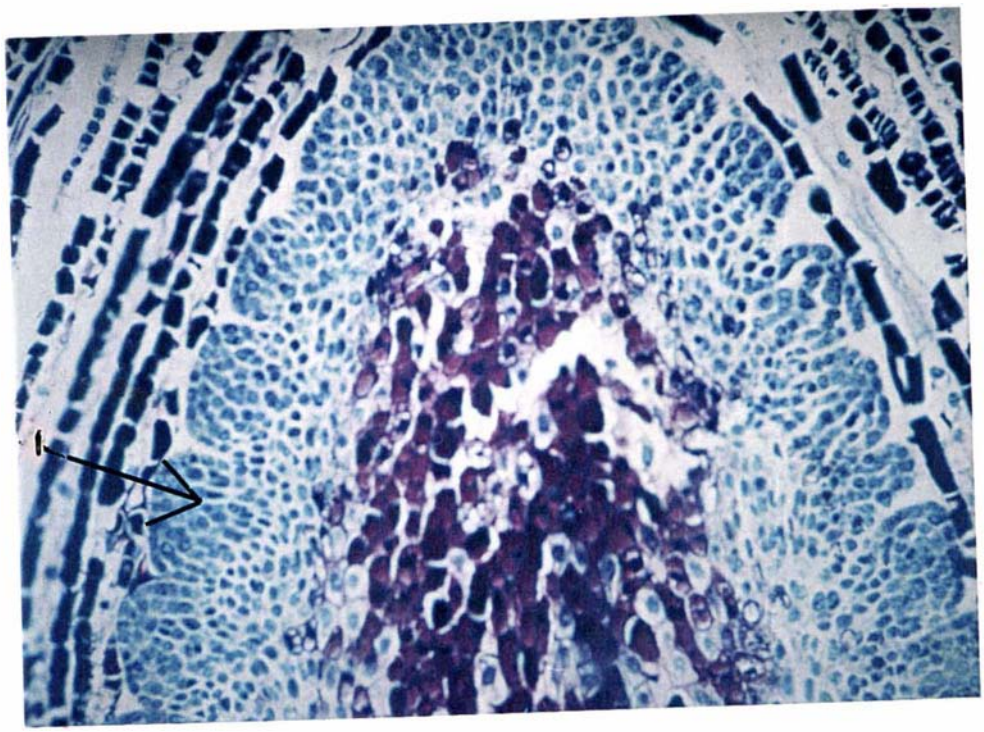



Fig 2.7. A median longitudinal section of a developing pollen cone bud collected in late February 1992, showing the acropetally initiated microsporophyll (arrow 1 ) beneath the apex, and the darkly stained pith cells. x160

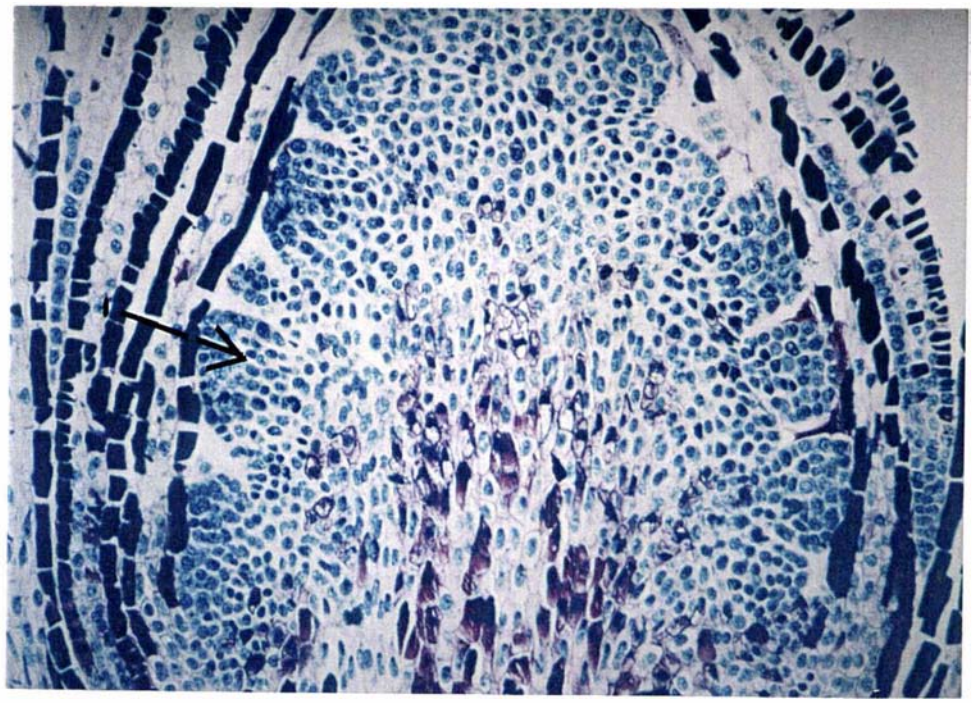


Fig 2.8. A median longitudinal section of a developing pollen cone bud collected in mid-March 1992, showing that the pollen cone bud apex was diminished by the initiated microsporophyll (arrow 1). Asymmetrical growth of the microsporophyll had begun to turn microsporophylls upwards. x160

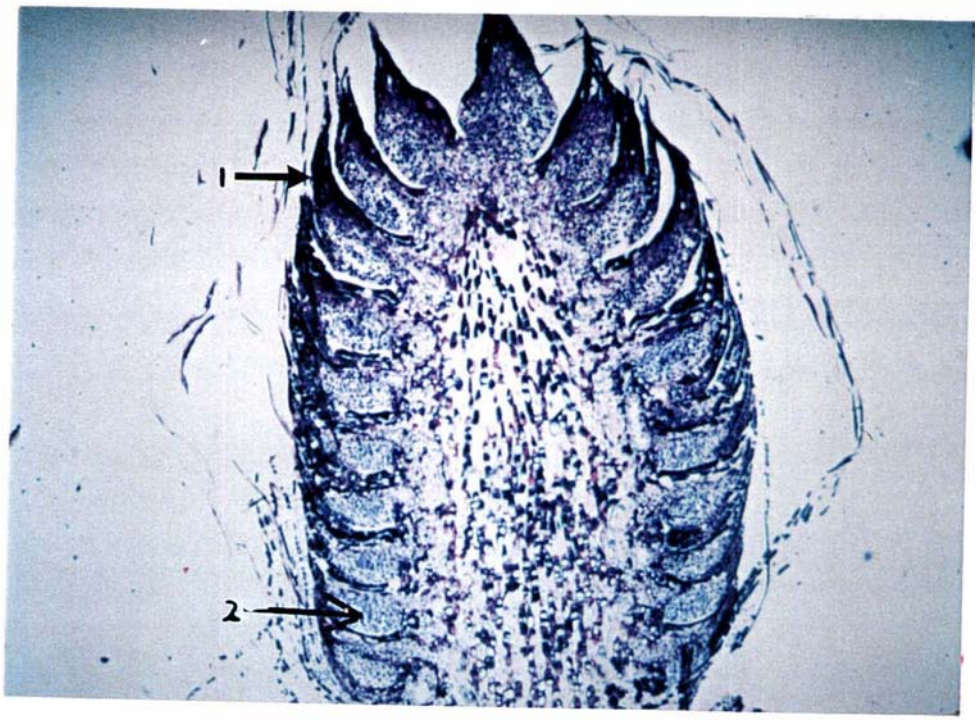



Fig 2.9. A near median longitudinal section of a pollen cone bud, after the completion of microsporophyll initiation in late April 1992, showing asymmetrical growth resulting in an upward turning of the microsporophylls (arrow 1). The sporogenous initials have been laid down in the abaxial part of some of the oldest microsporophylls (arrow 2). x40

The upper epidermis (adaxial surface) of the microsporophylls at this stage (arrow 1, Fig 2.10) consisted of broadly elongated cells which underwent frequent anticlinal divisions. Their outer walls were cutinised and most of them were filled with a uniformly suberised material which persisted even to the time of pollen shedding. Only the cells at the abaxial surface, marking the line of dehiscence, were free from suberization (arrow 2, Fig 2.10). The newly formed microsporangium with a wall of 4-5 cell layers (arrow 3, Fig 2.10) and sporogenous cells inside the wall were well defined at this stage. The tapetum layer could not be distinguished from other wall layers. The sporogenous cells were larger in diameter, had a denser cytoplasm and stained darker than the surrounding cells. They formed a compact mass with no apparent intercellular spaces among them (arrow 4, Fig 2.10). At the morphological level, there were two well defined microsporangia formed on the underside of each microsporophyll.

By late May, the sporogenous cells had divided in several planes to form a mass of pollen mother cells (PMC), each with dense cytoplasm and a large granular nucleus. They were also much larger than the wall layer cells (Fig 2.11). The dividing of a pollen mother cell was noted (arrow 1, Fig 2.11). These pollen mother cells were surrounded by a microsporangial wall layer two to three cells thick. The innermost layer next to the pollen mother cells were presumed to be the tapetum. It completely surrounded the PMCs. The tapetal cells were smaller than the PMCs, but their cytoplasm was strongly stained, indicating active metabolism of the cells at this stage (arrow 2, Fig 2. 11).

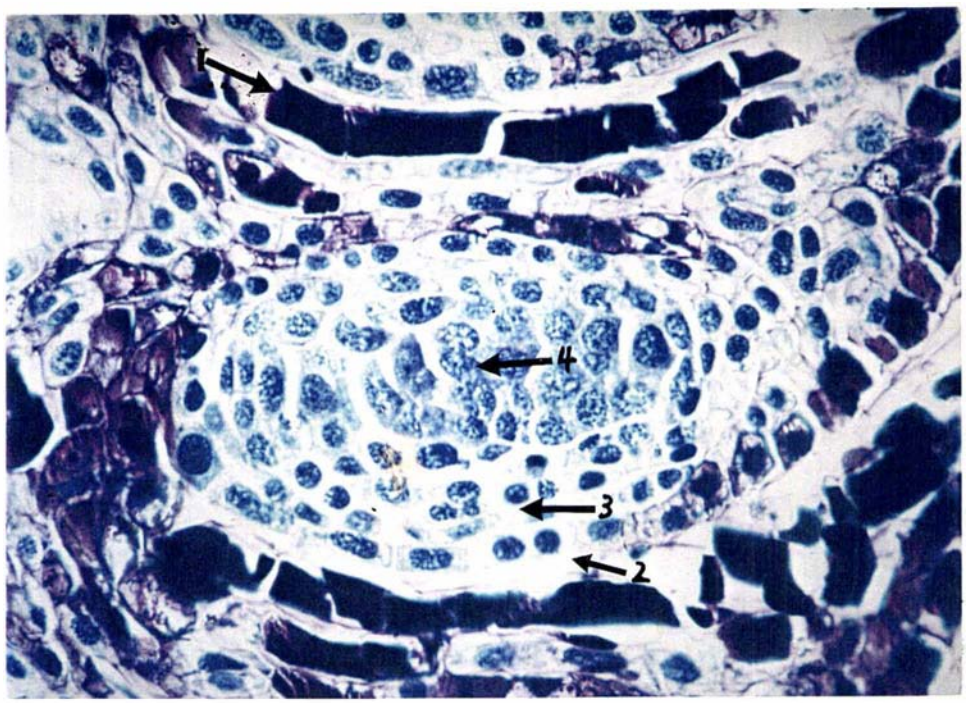



Fig 2.10. A higher magnification of Fig 2.9, showing the upper epidermis (adaxial surface) (arrow 1) cells filled with suberised material and the dehiscence line in the normal abaxial surface cells with no cutinised wall or suberised material (arrow 2). The closely gathered sporogenous cells (arrow 4) are surrounded within the microsporangium by a wall of 4-5 cell layers (arrow 3). x400

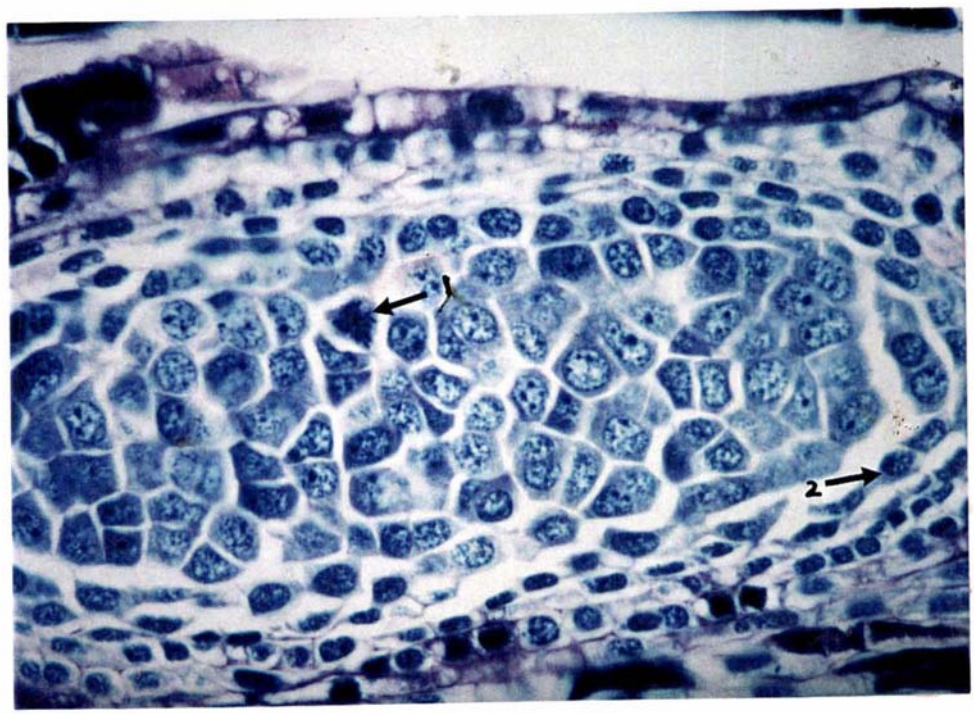



Fig 2.11. A longitudinal section of a microsporophyll, collected in late May 1992, showing the pollen mother cells within the microsporangium surrounded by the tapetal layer (arrow 2) and the outside wall layer cells, One pollen mother cell was in the process of dividing (arrow 1). x400

Closer examination of some of these pollen mother cells under high power showed that these pollen mother cells had probably entered the prophase stage of meiosis. The nucleus was larger than the previous stage, the chromosomes appeared as very fine strands and there was some suggestion of pairing of homologous chromosomes (arrow 1, Fig 2.12). Chromosomes were arranged randomly throughout the nucleus and nucleoli remained large and distinct (arrow 2, Fig 2. 12). Pollen mother cells seemed to be separated from each other by extra-cellular spaces (arrow 3, Fig 12). By May 27, chromosomes had become very distinct and indications of pairing was more evident (arrow 1, Fig 2.13), suggesting that the pachytene stage of prophase I had started in the pollen mother cells. Spaces between pollen mother cells were more obvious (arrow 2, Fig 2.13). The nature of this separations was further investigated under transmission electron microscope and is discussed later.

By mid-June, pollen mother cells had reached the late pachytene stage of prophase I. Paired chromosomes had contracted as thick, fuzzy strands, clumped in a tangled mass filling only a portion of the nucleus and covering the nucleolus (arrow 1, Fig 2.14). At this stage, pollen mother cells had also accumulated a number of colourless granules which had the appearance of starch grains (arrow 2, Fig 2.14). Subsequent development from this stage was very rapid. Sixteen days later, on July 2, pollen cones collected from the same lateral shoot at different positions showed both microspore tetrads and mature pollen grains.

Pollen cones collected from the upper part of the shoot tips, within 20 mm from the shoot apex, showed that the two meiotic divisions were completed; the resulting microspore tetrads (arrow 1, Fig 2.15; Fig 2.16) appeared inside the semi-degraded pollen-sac. The intact tapetal layer had disappeared at this stage; only isolated tapetal cells with starch grain-like granules remained along the degraded pollen-sac wall (arrow 2, Fig 2.15). The mid-layer cells outside the tapetal cells had almost completely disappeared. They are believed to be degraded during the enlargement of the pollen-sac (arrow 3, Fig 2. 15). In pollen cones collected from the lower part of the shoot tips, *i.e.* more than 50 mm away from the shoot apex, the pollen mother cells had completed meiosis, and well developed pollen grains with two air bladders had been formed (Fig 2.17).

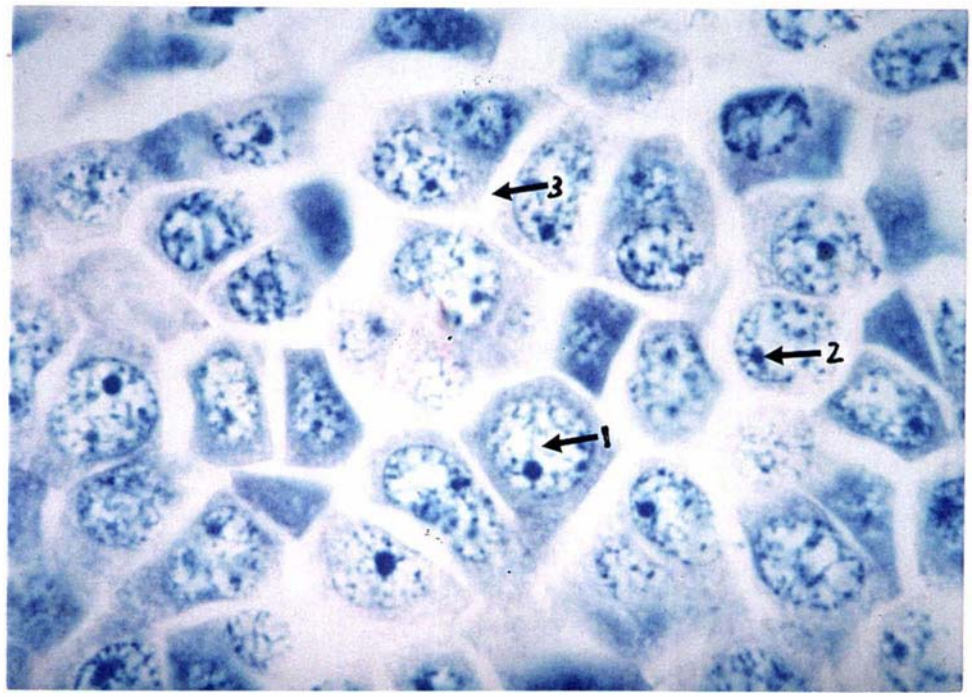



Fig 2.12. A higher magnification of Fig 2.11, showing the larger nucleus, and the early prophase stage of meiosis in PMCs. Chromosomes appear as fine strands and some pairing has occurred (arrow 1), the nucleoli remain large and distinct (arrow 2). Cell walls are shown (arrow 3). x1000

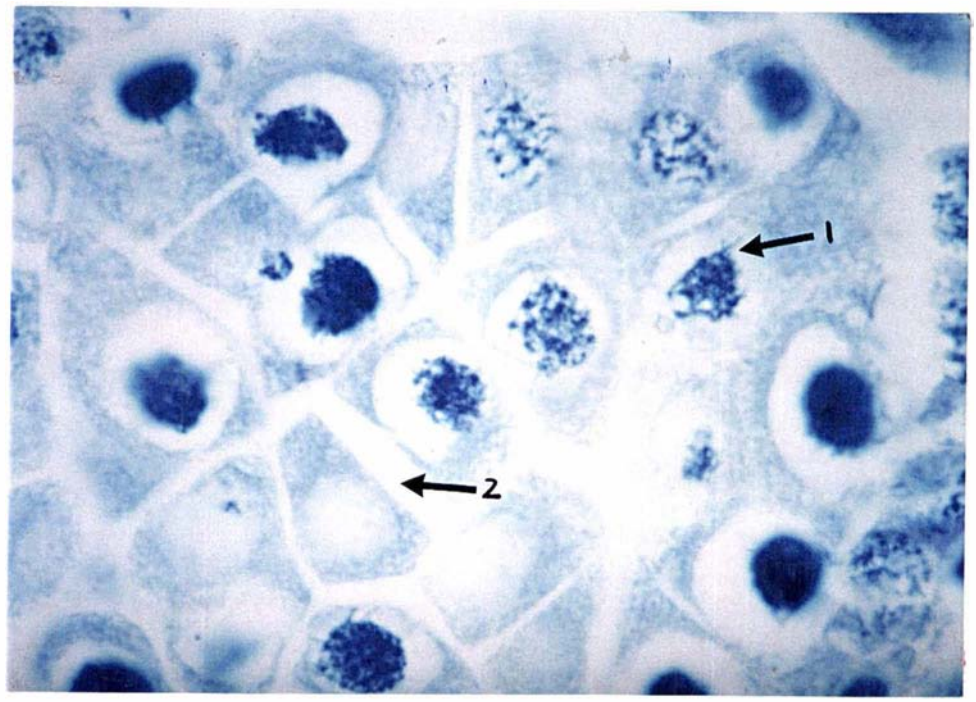



Fig 2.13. A longitudinal section of a microsporophyll, collected at the end of May 1992, showing the early pachytene stage of meiosis in PMCs. Chromosomes are thicker and pairing is more evident (arrow 1), with the cell wall becoming thicker (arrow 2). x1000

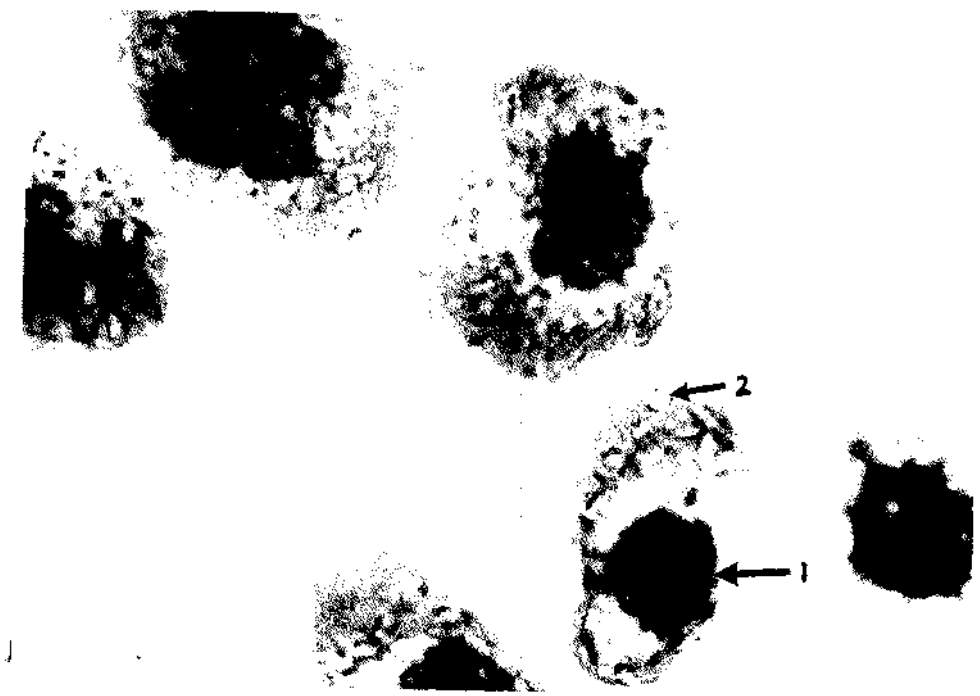



Fig 2.14. A longitudinal section of a microsporophyll, collected in mid-June 1992, showing the characteristic pachytene stage in PMCs. Clumping of chromosomes at one side of the nucleus is common at this stage. Paired chromosomes contract as thick fuzzy strands (arrow 1). Some starch-like grains are evident in PMCs (arrow 2). x1000

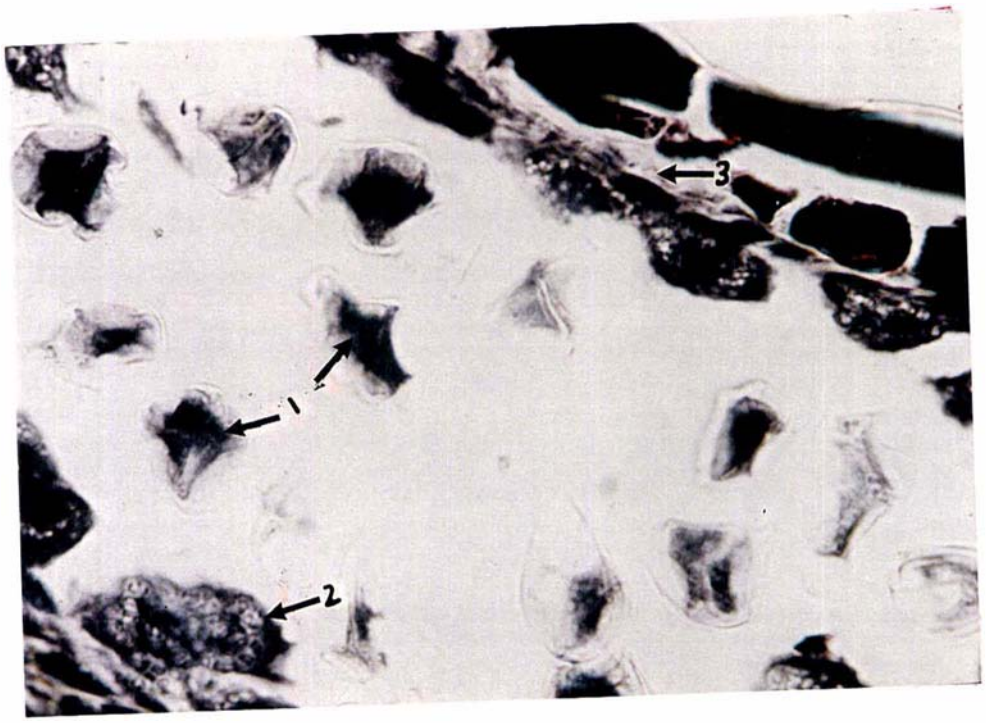



Fig 2.15. A longitudinal section of a microsporophyll, collected in early July 1992, showing microspore tetrads (arrow 1). The wall layers of the microsporangium had almost disappeared (arrow 3), leaving only the tapetal cells with some starch grains. (arrow 2). x160

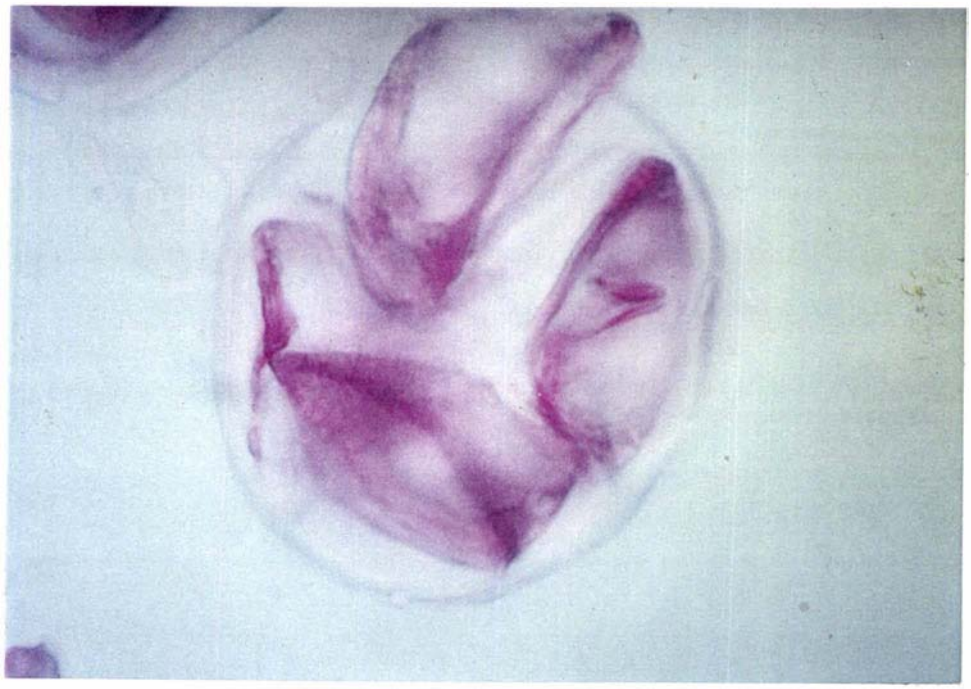


Fig 2.16. A freshly prepared section obtained by a microsporophyll squash showing a well formed microspore tetrad. The microsporophyll was collected from pollen cone buds located on the distal zone of the shoot axis in early July 1992. The section was stained with basic fuchsin. x1000.

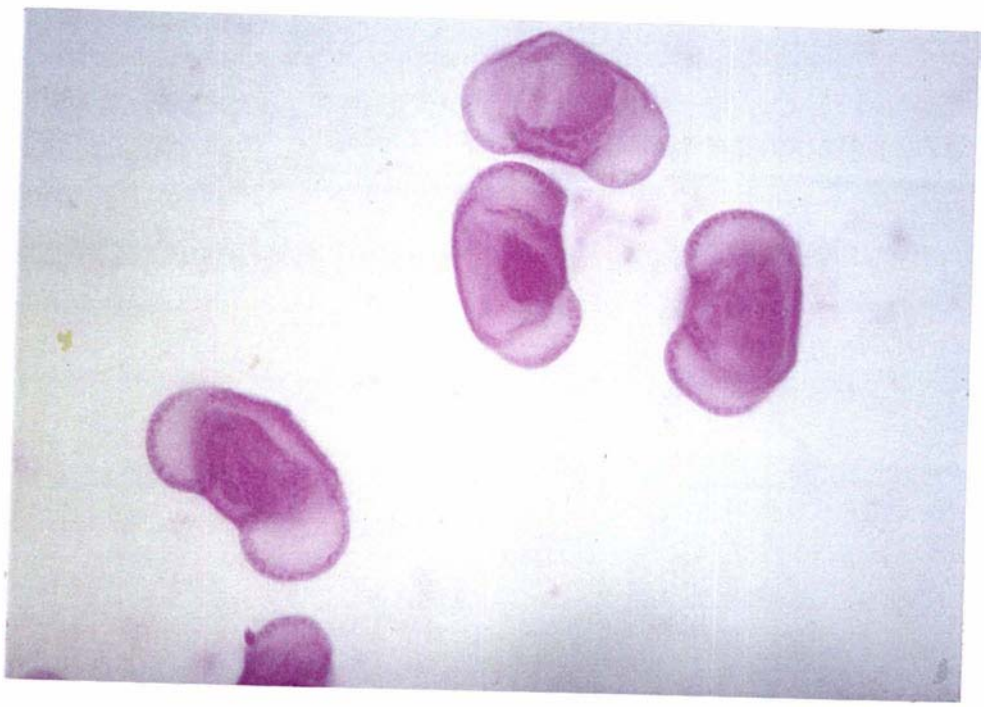


Fig 2.17. A freshly prepared section obtained by a microsporophyll squash, showing a mature pollen grain. The microsporophyll was collected from the basal zone of the pollen cone bud in early July 1992. The section was stained with basic fuchsin. x160.

## 2.3.3. ANATOMICAL STUDY OF THE MALE CONE DEVELOPMENT IN *Pinus radiata* RESULTS FROM TRANSMISSION ELECTRON MICROSCOPY

Ultrastructural changes that occurred at several selected stages during microsporogenesis in *Pinus radiata* were studied using transmission electron microscopy. This study examined the nature of the intercellular connections among pollen mother cells (PMCs) and tapetal cells, the reorganisations of the subcellular organelles of PMCs and tapetal cells, and the occurrence of callose walls among the PMCs and tapetal cells during the meiotic process; changes were studied from the time when the microsporangium had been formed (29/4/92), until the late pachytene stage of meiosis for pollen mother cells (16/6/92).

In sections prepared from microsporangium tissue collected on 29/4/92, showing the larger central sporogenous cells (arrow 1, Fig 2.18) and the surrounding smaller tapetal cells (arrow 2, Fig 2.18), more than one, usually two or three nucleoli were present inside the nucleus of both cell types. (arrow 3, Fig 2. 18). There were some vacuoles, plastids (arrow 4, Fig 2.18) and mitochondria seen in both sporogenous cells (arrow 1, Fig 2.19) and tapetal cells (arrow 1, Fig 2.20). Well developed plasmodesmata connections more frequently occurred between tapetum cells than among the sporogenous cells (arrow 2, Fig 2.20), but there was a clear plasmodesmatal connection between two sporogenous cells, apparently showing contact between rough endoplasmic reticulum (RER)(arrow 2, Fig 2.19). Cell membranes were well defined, as was the nuclear envelope. Free ribosomes were abundant (arrow 3, Fig 2.19; arrow 3, Fig 2.20) and the rough endoplasmic reticulum (RER), although not abundant, was evenly dispersed throughout the cytoplasm in both tissues. Dictyosomes (arrow 4, Fig 2.19; arrow 4, Fig 2.20) were also seen in both tissues.

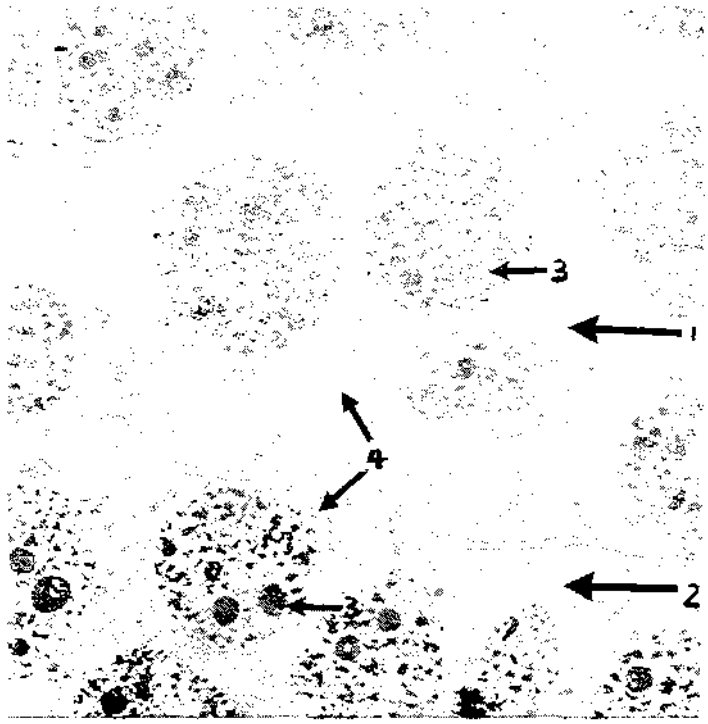



Fig 2.18. Part of a microsporangium from a microsporophyll collected in late April 1992, showing the surrounding tapetal cells (arrow 2) and the central sporogenous cells (arrow 1). Multiple nucleoli were present inside the nucleus of both the sporogenous cells and the tapetal cells (arrow 3). A number of plastids can be seen in both sporogenous cells and tapetal cells (arrow 4). x2345

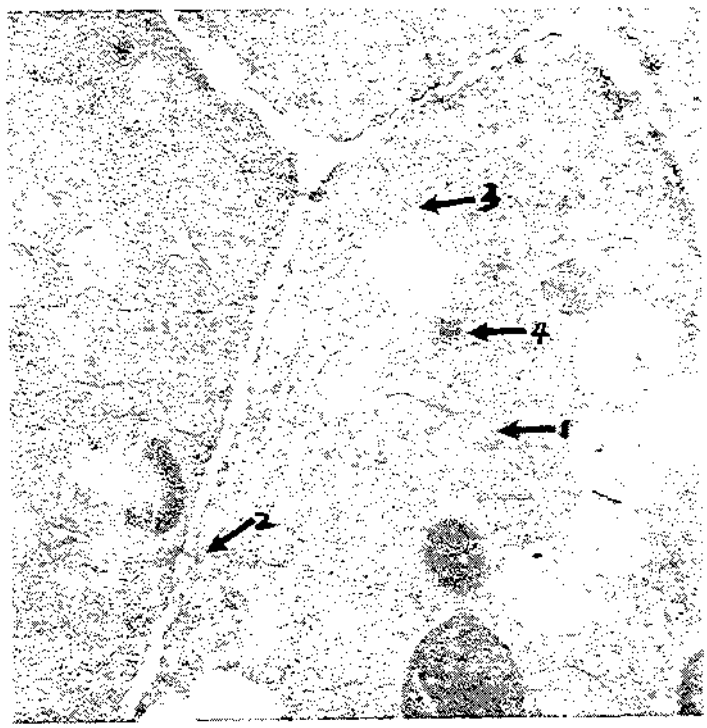



Fig 2.19. A higher magnification of Fig 2.18, showing part of three sporogenous cells each containing numbers of mitochondria (arrow 1) and free ribosomes (arrow 3). One plasmodesma strand is seen apparently connecting with a rough endoplasmic reticulum (RER) channel (arrow 2). A dictyosome is also evident (arrow 4). x15330

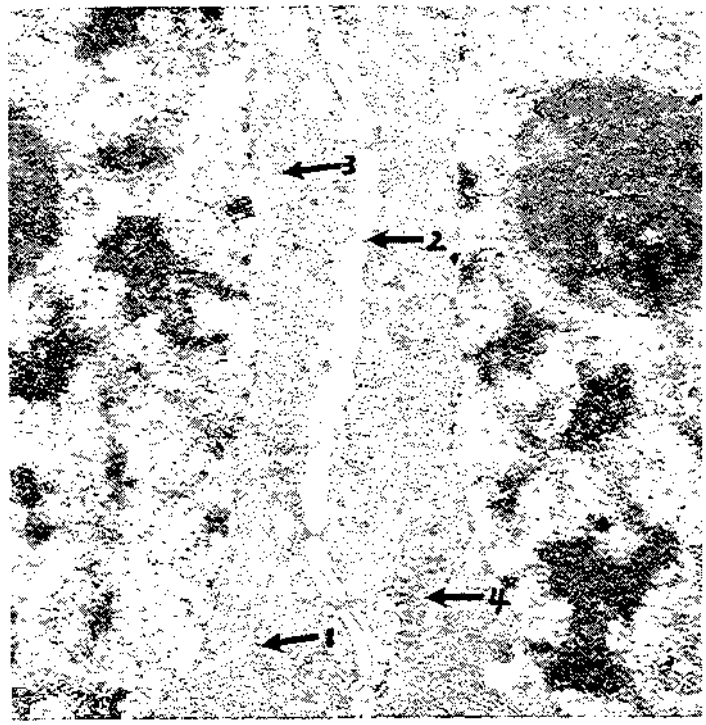



Fig 2.20. A higher magnification of Fig 2.18, showing part of two tapetal cells with much more frequent plasmodesma connections between them (arrow 2). Rich number of ribosomes (arrow 3) and some dictyosomes (arrow 4) are evident. Mitochondria are also evident (arrow 1). x15330

By 20/5/92, after the sporogenous cells had differentiated into PMCs, the general shape of the PMCs was distinctly different from that of the tapetal cells. PMCs were hexagonal-pentagonal (arrow 1, Fig 2.21), while the tapetal cells were rectangular-trapezoidal (arrow 2, Fig 2.21). No evident intercellular connections were seen between a tapetal cell and the PMCs (Fig 2.22). The number of the plastids and mitochondria remained abundant, as did the free ribosomes (Figs 2.22, 2.23, 2.24). The nuclear envelope of the PMCs was well defined and the contact of the rough endoplasmic reticulum (RER) to the nuclear envelope was evident (arrow 1, Fig 2.23), indicating the existence of the pathway for genetic information between the nucleus and the cytoplasm. Cell membranes of the PMCs were still visible, but an unstained wall of a probable callosic nature had blocked the plasmodesmata connections among the PMCs and the connections with the tapetal cells (arrow 1, Fig 2.24).

Plasmodesma were rarely seen at this stage, but more vacuolated structures similar to autophagic vacuoles started to appear (arrow 3, Fig 2.23; arrow 2, Fig 2.24).

By 27/5/92, when PMCs started their pachytene stage, some significant ultrastructural changes had taken place among the PMCs. More and larger autophagic vacuoles appeared (arrow 1, Fig 2.25), and previously well defined cell walls appeared thinner and less distinctive (arrow 2, Fig 2.25). A number of mitochondria gave the appearance of being engulfed by the autophagic vacuoles (arrow 1, Fig 2.26). Most plastids examined were encircled by the dilated rough endoplasmic reticulum (arrow 2, Fig 2.26; arrow 1, Fig 2.28; arrow 1, Fig 2.29), and there were more dilated RER, and more plastid and dictyosomes present in PMCs than seen in the previous stage close to 30 days before (Figs 2.26, 2.27, 2.28, 2.29). The appearance of the cell wall was different from earlier stages and showed a suggestion of reduced rigidity. It appeared to be callosic in nature, as judged by staining properties and uneven thickening. There were signs that the plasmodesmata connections among these PMCs were blocked by this callosic wall (arrow 2, Fig 2.29). There was little change in the number of mitochondria or of free ribosomes.

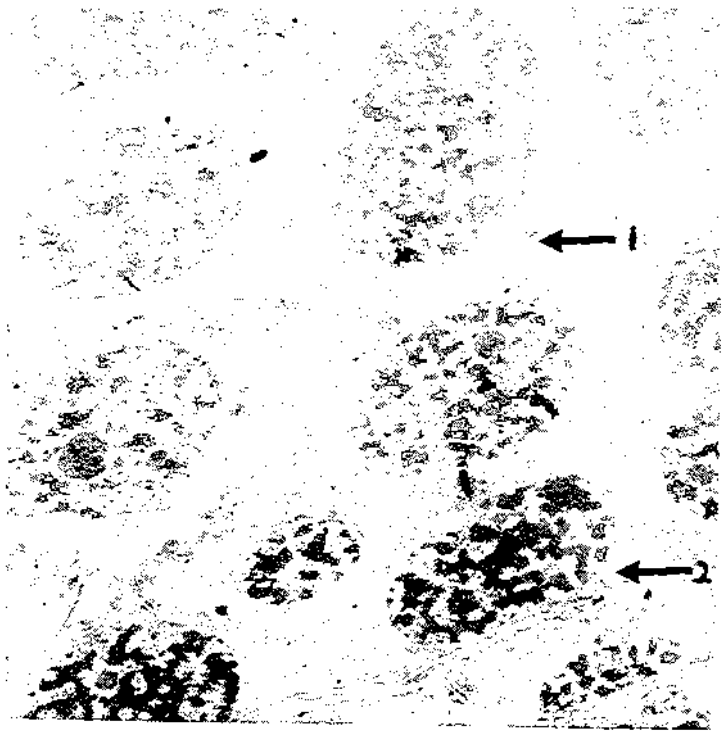



Fig 2.21. Part of a microsporangium from a microsporophyll collected in late May 1992, showing distinctly different shapes between the pollen mother cells (arrow 1) and the tapetal cells (arrow 2). x3413

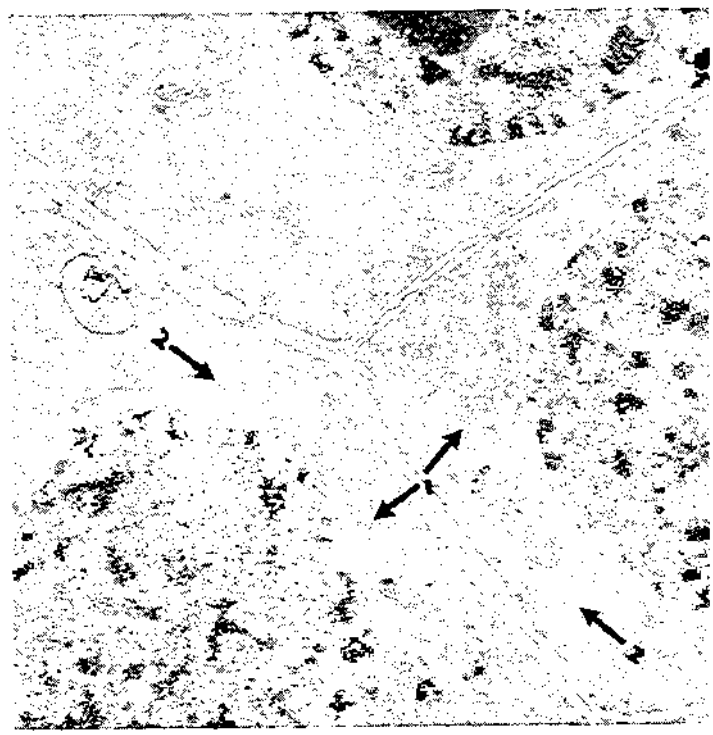


Fig 2.22. Part of a microsporangium from a microsporophyll collected in late May 1992, showing one tapetal cell (lower left corner) and two pollen mother cells (upper right corner). Plastids (arrow 1), mitochondria (arrow 2) and free ribosomes are abundant. x11200

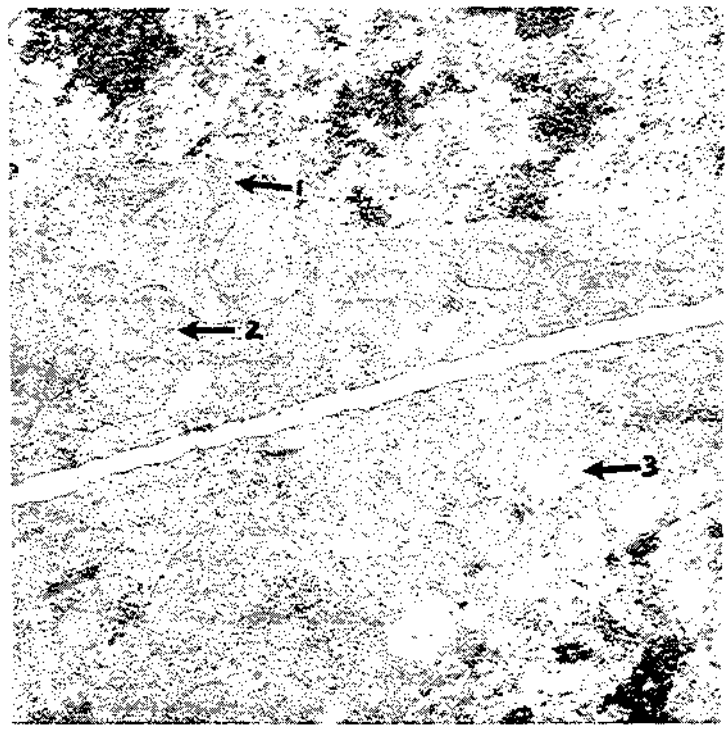



Fig 2.23. A higher magnification of Fig 2.21, showing part of the two pollen mother cells. The contact of RER to the well defined nuclear envelope is evident (arrow 1). Abundant mitochondria are also evident (arrow 2). Autophagic-like vacuoles are also apparent (arrow 3). x15330

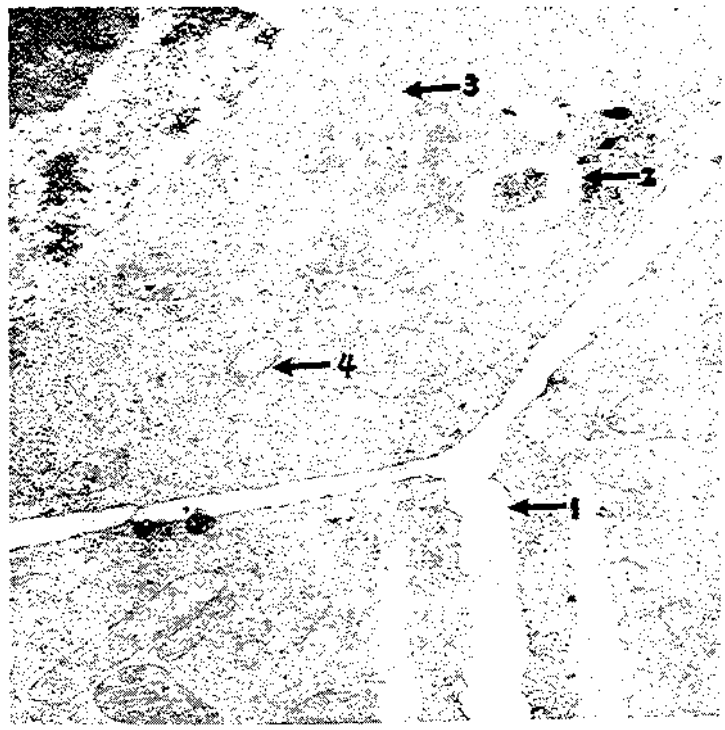



Fig 2.24. A higher magnification of Fig 2.21, showing parts of three pollen mother cells. Unstained callosic wall material has apparently blocked the intercellular connections among the cells (arrow 1). Autophagic-like vacuoles are evident (arrow 2). The number of plastids (arrow 3) and mitochondria (arrow 4) are abundant. x15330

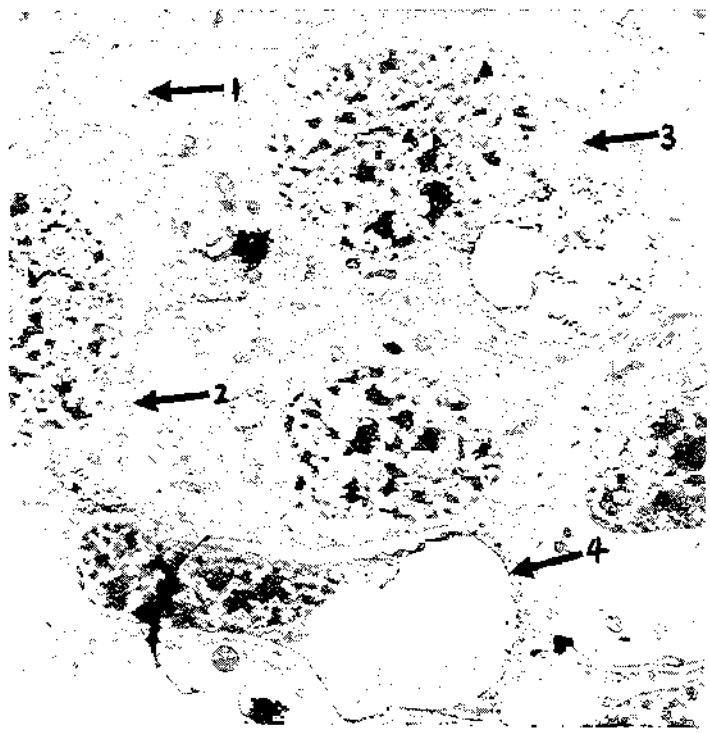



Fig 2.25. Part of a microsporangium of a microsporophyll collected at the end of May 1992, showing autophagic vacuoles (arrow 1) and the relatively thinner cell wall (arrow 2), and also showing different features of the central pollen mother cells (arrow 3) and the surrounding tapetal cells (arrow 4). x3413

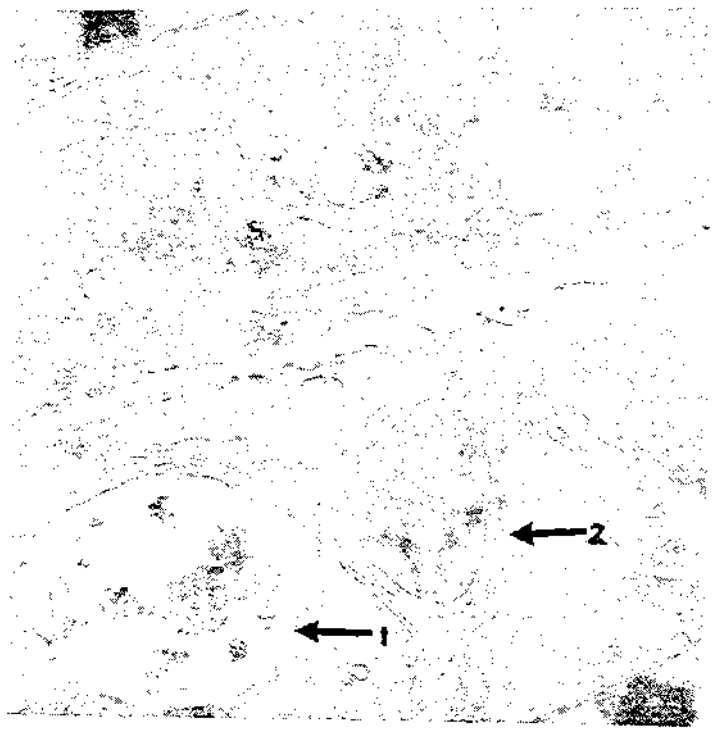



Fig 2.26. Higher magnification of Fig 2.25, showing part of the pollen mother cell. Mitochondria are seen to be engulfed by autophagic vacuoles (arrow 1) and the plastids are encircled by the dilated rough endoplasmic reticulum (arrow 2). x15330

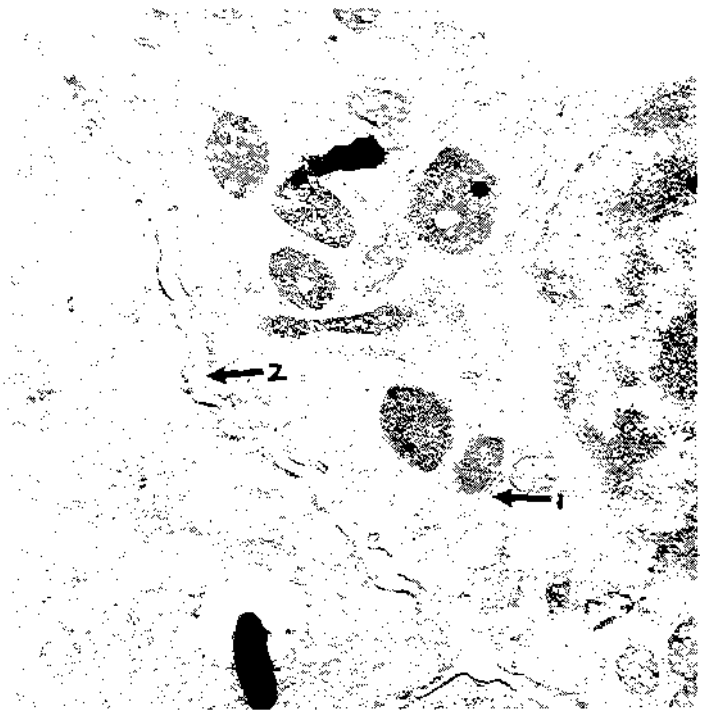



Fig 2.27. Higher magnification of Fig 2.25, showing part of the pollen mother cell. More dilated rough endoplasmic reticulum (RER) is seen (arrow 1). The cell wall becomes less distinctive (arrow 2). x15330

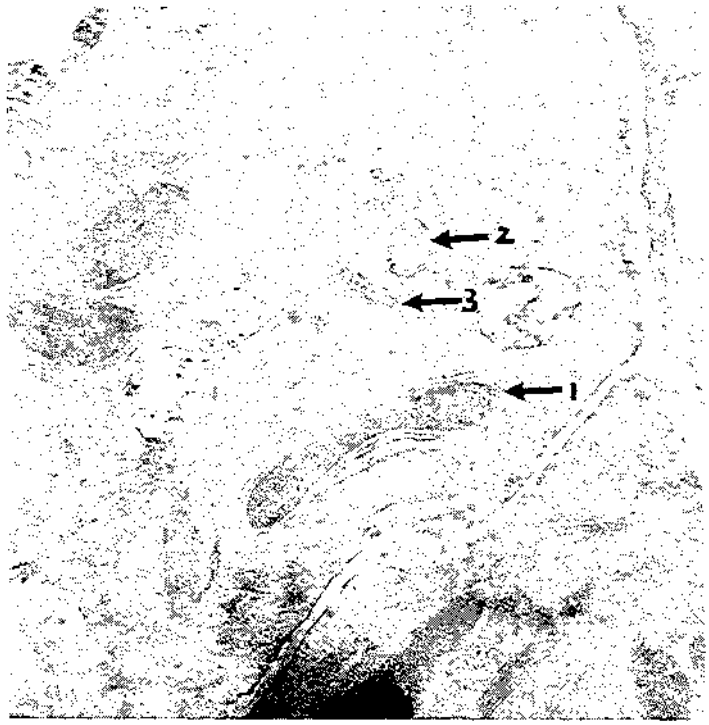



Fig 2.28. Higher magnification of Fig 2.25, showing part of the pollen mother cell. The plastids are encircled by the dilated RER (arrow 1), and mitochondria (arrow 2) and dictyosomes (arrow 3) are evident. x15330

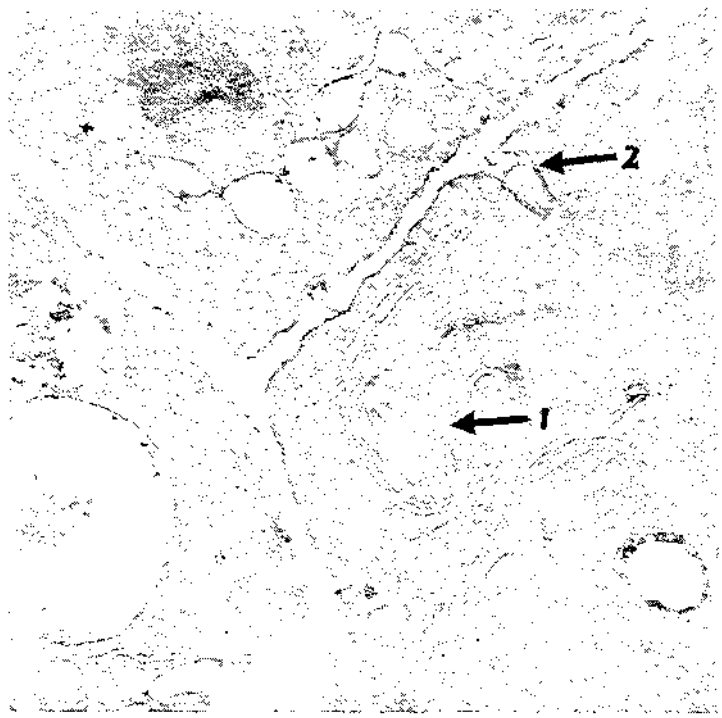



Fig 2.29. Higher magnification of Fig 2.25, showing part of the pollen mother cell, the plastids are encircled by the dilated RER (arrow 1) and the plasmodesmata connection is blocked by the callosic wall (arrow 2). x15330

By 3/6/92, when the PMCs were at about mid-pachytene stage, both tapetal cells and PMCs had apparently undergone a modification from a structurally meristematic to a hypersecretory appearance (Figs 2.30, 2.31, 2.32). The tapetal cells became radially flattened, nuclei and cytoplasm became intensely basophilic and dilation of the RER system was observed to be extreme throughout the cytoplasm (arrow 1, Fig 2.30). Osmiophilic granules and globules appeared on the radial surface of the tapetal cells (arrow 2, Fig 30). Channels formed by extreme dilation of ER occurred adjacent to the nucleus (arrow 1, Fig 2.31) and near the cell surface (arrow 1, Fig 2.32). These channels contained fibrillar material and appeared to open to the extensive spaces in the cytoplasm. It is possible that these areas are evidence of cytoplasmic degradation. Cell membranes were evident on the anticlinal surfaces of the tapetal cells but from examinations of many sections, no cytoplasmic connections between these tapetal cells were seen. Dilated portions of the RER/ER containing fibrillar flocculant material were confluent with envelopes of the autophagic vesicles or the broad and channel-like loculus (arrow 2, Fig 2.32). Free ribosomes were observed arranged into groups (polyribosomes) (arrow 3, Fig 2.32). There was a reduction in the relative density of plastids, mitochondria and dictyosomes (Figs 2.30, 2.31, 2.32). The callosic wall appeared to have blocked any possible intercellular connections between the tapetal cells (arrow 4, Fig 2.32). At the same time, PMCs also showed some hypersecretory features; broad channels formed by extremely dilated RER were evident (arrow 1, Fig 2.33). The dilated portions of the RER containing fibrillar flocculant material were also seen confluent with envelopes of the autophagic vesicles (arrow 2, Fig 2.33). The density of ribosomes decreased. The free ribosomes were observed to be arranged into polyribosomes in a similar way to the finding for tapetal cells (arrow 3, Fig 2.33). There was also a reduction in the relative densities of plastids, mitochondria and dictyosomes. A thick callosic wall had apparently isolated the PMCs from the tapetal cells (arrow 4, Fig 2.33). In comparison to tapetal cells, the cytoplasm of PMCs was less basophilic. This may indicate that the tapetal cells are more hypersecretory than PMCs.

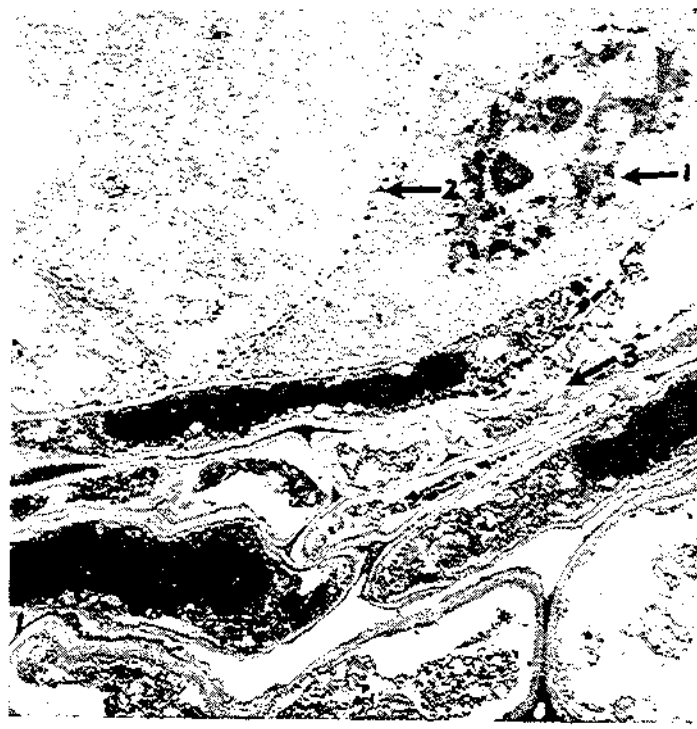



Fig 2.30. Part of the microsporangium from the microsporophyll collected in early June 1992, showing a hypersecretory tapetal cell with the extremely dilated RER system (arrow 1), the appearance of the osmiophilic granules (arrow 2) and the degraded middle wall layer cells (arrow 3). x3413

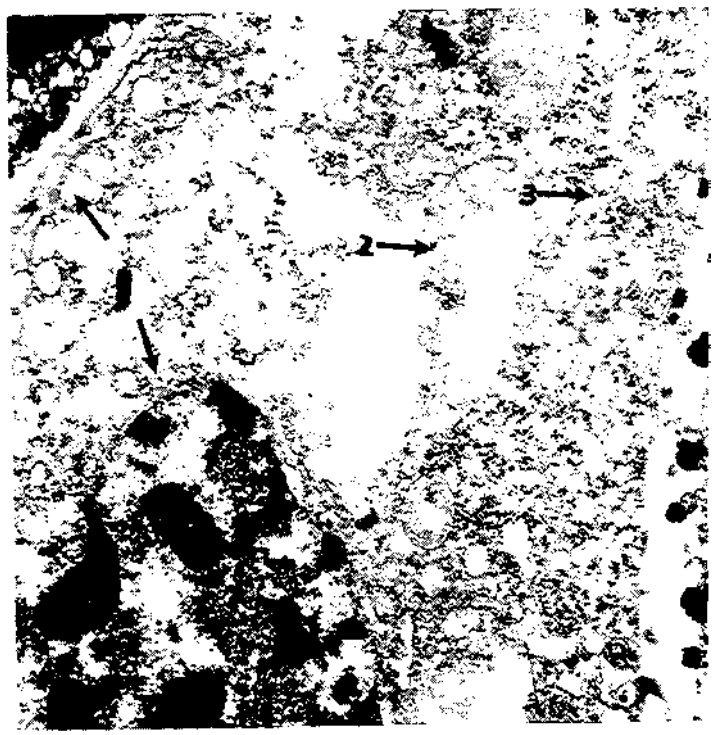



Fig 2.31. Higher magnification of Fig 2.30, showing part of the tapetal cell. Channels formed by extreme dilation of ER occurred adjacent to the nucleus and near the cell surface (arrow 1) are seen. Dilated portions of the ER/RER are confluent with envelopes of the autophagic vesicles or the channel-like loculus (arrow 2). Free ribosomes are arranged into groups (arrow 3). x15330

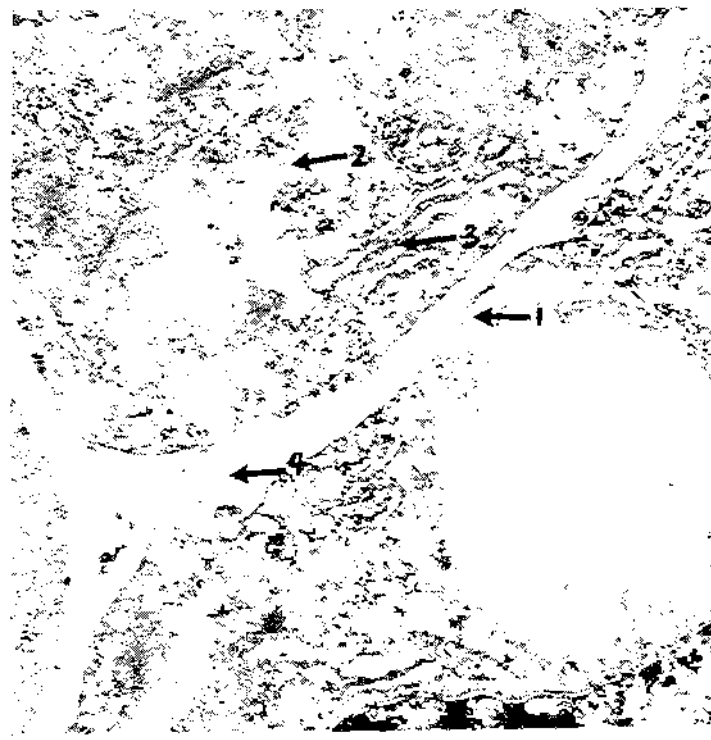



Fig 2.32. Higher magnification of Fig 2.30, showing part of the tapetal cell. Channels formed by extreme dilation of ER /RER are clearly seen adjacent to the cell surface (arrow 1), and confluent with the channel-like loculus (arrow 2). Free ribosomes are clearly arranged into groups (arrow 3). The callosic wall appears to block any possible intercellular connections (arrow 4). x15330

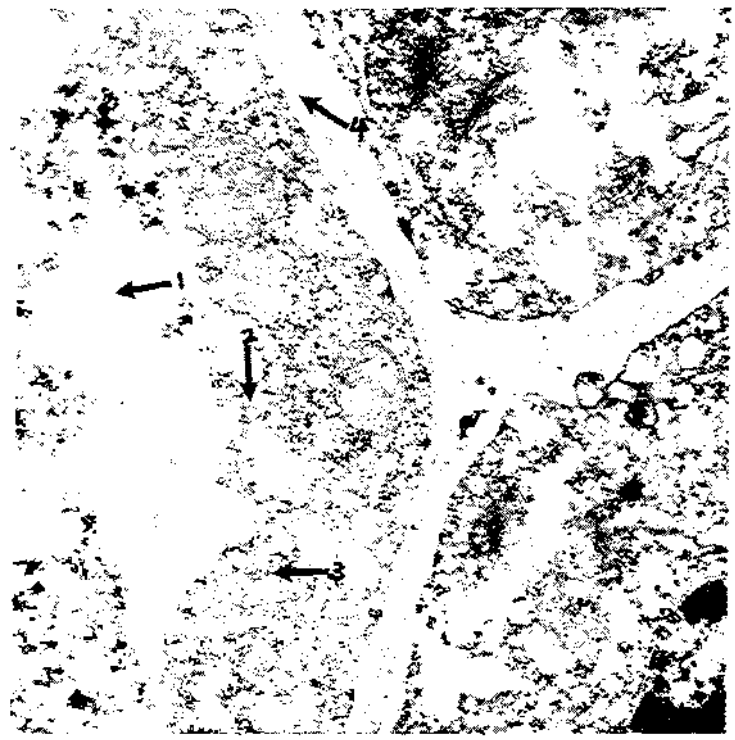



Fig 2.33. Part of a microsporangium from a microsporophyll collected in early June 1992, showing parts of two tapetal cells (right) and one pollen mother cell (left). Broad channels formed by extremely dilated endoplasmic reticulum (ER) and rough endoplasmic reticulum (RER) are evident (arrow 1), the dilated portions of the ER/RER are also seen confluent with envelopes of autophagic vesicles (arrow 2). Free ribosomes are seen arranged into groups (arrow 3). A thick callosic wall has apparently isolated the pollen mother cells from the tapetal cells (arrow 4). x15330

By 16/6/92, when the PMCs entered the end of the pachytene stage of prophase I, a prominent invagination occurred along the cytoplasm membrane of the PMCs beneath the cell wall (arrow 1, Fig 2.34). Some osmiophilic granules and globules were seen along these invaginations (arrow 1, Fig 2.35). Increased surface area to facilitate absorbing of nutrients secreted by the tapetal cells seemed to be a conceivable function of this invagination. The callosic wall of the PMCs was evident (arrow 2, Fig 2.35). The density of ribosomes was reduced again compared to the earlier stage. The numbers of plastids and mitochondria were also reduced significantly (Figs 2.34, 2.35). These ultrastructural modifications appeared to indicate that the cell metabolism of the PMCs had undergone a significant change in preparation for the switch over from sporophytic development to gametophytic development, which started with the two continuous reduction divisions around late June.

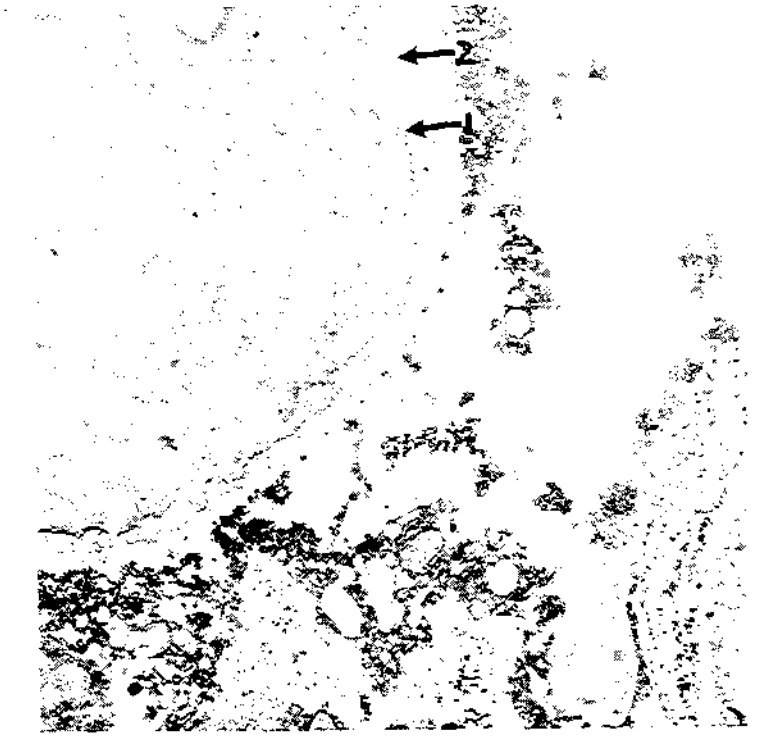



Fig 2.34. Section prepared from tissue collected in mid-June 1992, showing a pollen mother cell with an invaginated cytoplasmic membrane (arrow 1) coated with a thick callose wall (arrow 2). x3413

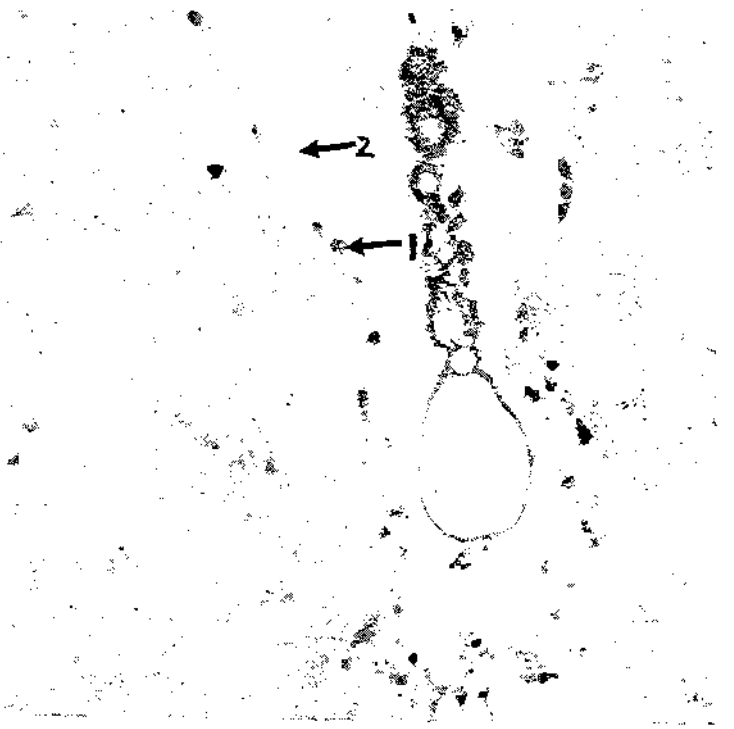



Fig 2.35. Part of two pollen mother cells from a microsporophyll collected in mid-June, showing invagination of the cytoplasmic membrane. Some osmiophilic granules and globules are seen along these invaginations (arrow 1), and fewer condensed ribosomes, plastids and mitochondria are seen at this stage. The callosic wall of these two pollen mother cells is evident (arrow 2). x15330

# 2. 4. DISCUSSION

## 2.4.1. THE TIMING OF THE DEVELOPMENTAL EVENTS DURING THE MALE CONE DEVELOPMENT IN *Pinus radiata*, AND ITS RELATIONSHIP WITH ENVIRONMENTAL FACTORS.

Male cones of *Pinus radiata* are initiated by the terminal buds of the subordinate shoots. These subordinate shoots exhibit a pre-determined growth pattern similar to the leading shoots, but the structural components of the shoots are different. The components of the new season's subordinate shoots are initiated in the previous growing season in a bud form. It has been reported that pollen cones of *P. radiata* growing in New Zealand are initiated in spring when the first short shoots are formed (Bollmann and Sweet, 1976). The timing of the initiation of the male cone in *P. radiata*, growing at a site near Canberra, Australia has been given by Cremer (1992). His study indicated that the cataphylls that were due to bear the male cones initiated between about October-December. The actual short-shoot initials in the axils of these cataphylls became visible in January, and their differentiation, *i.e.* their commitment to either foliage or male cone production, occurred in early February .

The present study could not detect any signs of short shoot primordia which will later develop into pollen cones on a subordinate shoot, collected on 19/11/91, apart from an apical meristem encased in a series of broad, sclerified achlorophyllous sterile cataphylls (Fig 2.3). The earliest appearance of the axillary apices which will later on form the first short shoot was seen on the subordinate short shoot terminal bud collected in early summer (5/12/91), under the apparently favourable growing conditions at Rotorua. These earliest axillary apices were predetermined to develop into the first pollen cones, but differentiation into pollen cone primordia does not occur until three or four axillary apices are formed above them on the axis of the subordinate shoots. In this study, the differentiation appeared to be occurring at the collection date of 15/1/92 (Fig 2.5). Before this date, determination of axillary buds as male cone buds or vegetative dwarf shoot buds could not be made with confidence. An increasing frequency of supply of the shoot tips obtained over the time period, from early December to early January might have allowed better determination of cone bud initiation time, but other factors undoubtedlly need to be considered, such as the positions of the male LSTB on the crown, exposure to the direction of sunshine and genetic factors.

Mid-January to February is in the middle of New Zealand summer, which is the warmest and often the driest time of the year (Bollmann and Sweet, 1979). The warm temperature provides favourable conditions for the development of pollen cones. By late February and early March, the initiation of microsporophylls had finished. From then, the sporogenous tissue continued to develop continuously until the shedding of the mature pollen. The whole process took only about five months. Once sporogenous cells had differentiated into PMCs and entered the meiosis process, it took six weeks for PMCs to complete the developmental process resulting in the formation of pollen grains. Of these six weeks, in the first four of them, PMCs were at the prophase stage, It appears to take a relatively long period of time for PMCs to prepare for reduction division, and the two continuous reduction divisions were then completed just within two weeks. As noticed in this study, on 16/6/92, PMCs were still at the late pachytene stage (Fig 2.14) but by 2/7/92, well defined tetrad and fully formed pollen grains were already present (Figs 2.15, 2.16, 2.17).

In Northern temperate pines in contrast, all axillary buds necessary for the differentiation of reproductive buds are initiated in late summer and autumn inside the LSTB before the LSTB becomes dormant through winter (Owens, 1985). They resume their development in the next spring. However, depending on the species, reproductive bud differentiation and development may pause at various climate-dependant points. For example, in *P.contorta,* PCB were differentiated and developed to near completion before winter (Owens and Molder, 1975). In *P.taeda* (Greenwood, 1980), *P.resinosa* (Duff and Nolan, 1958), and *P.ponderosa* (Gifford and Mirov, 1960), PCB differentiated and underwent considerable development before winter. Pollen cones in slash pine (*P.elliottii*) passed winter in the pollen mother cell stage and meiosis occurred during the end of winter and the beginning of next spring (Mergen, 1957).

In other gymnosperm species, a developmental break is also apparent. In some species of *Larix*, pollen cone buds had started their meiosis during autumn and stopped. They passed winter at the diplotene stage, and completed their meiosis by next spring (Ekberg, 1968). However in *P. monticola* (Owens and Molder, 1977), pollen cone buds differentiated but showed little development before winter. All microsporophylls were initiated but sporogenous tissue did not form before pollen cone buds became dormant in winter. Environmental or climatic conditions appear to be the major factor controlling the developmental timing of events leading to pollen production. The higher latitudes of the

Northern Hemisphere impose a cessation of development and break pollen cone development into two quite separate parts by a period of some 2-3 month's dormancy.

Pollen cone development in the milder climatic conditions of New Zealand is quite different when compared with developmental timing of Northern temperate pines. However a comparison of the two is still valid, since the organogenic sequence does not vary. By comparing the timetable of the pollen cone development between them, we can see that both "rate" or developmental timing of pollen cone differentiation and development, especially the meiosis event are strongly influenced by climate or other environmental cues such as daylength. Evidence reviewed here suggests that temperature is of greatest importance.

For example in northwest British Columbia, Canada, pollen cones of *P. monticola* only differentiate into the microsporophyll stage with no initiation of the sporogenous tissue before winter and pollen shedding does not occur until late the following June (Owens and Molder, 1979). On the other hand, in Baker County, Florida, a warmer and lower latitude area, pollen cones of *P. elliottii* would differentiate into pollen mother cells before they went into their winter dormancy, and pollen shedding will occur as early as the end of the following January (Mergen, 1957). *Pinus radiata* growing in its natural environment on the Pacific coast of California, sheds pollen in March after winter dormancy (Mirov, 1967). In New Zealand, pollen cones of *P. radiata* develop through winter and pollen shedding is in the middle of the winter (early July) according to this study, or around August-September according to Bollmann and Sweet (1976). Fountain and Cornford (1991) over a three year study period found the starting date for pollen release varied from early July to early August. Mirov had reviewed the environmental impact on pollen cone development in his book "*The Genus, Pinus*". He stated that in the temperature zone, pines shed their pollen during the season of the year designated as spring (Mirov, 1967). In Northwest British Columbia, Canada, spring is in June; in the coastal California, spring is in March and in a southern hemisphere country like New Zealand, it is in August-September. Mirov also pointed out that the closer to the Equator, the earlier pine pollen is shed. But he did not think that a changed photoperiod would affect the "flowering" of pines, he rather suggested that the involvement of thermoperiodicity was a major factor (Mirov, 1967).

Based on their studies on *P. sylvestris* and *P. palustris*, Sarvas (1962) and Boyer and Woods (1973) reached a similar conclusion that increased temperatures of the environment will shorten the total time to anthesis *i.e.* higher temperatures will cause earlier pollen shedding. To further emphasise the environmental (mainly temperature) impact on pollen cone development, an example of abnormal pollen cone development in *Larix*, growing in Sweden, is given. If the pollen cone buds did not have a dormancy period through winter, frequently no wall formation was seen after the completion of meiosis of the pollen mother cells. Eight microspores formed rather than four, and these microspores were regarded as non-functional ones (Ekberg, 1968). No literature however, has been found allowing comment on whether this occurs in pines or not.

The winter dormancy period appears to accelerate meiosis in these conifer species. Once pollen cones have completed winter dormancy, it takes only a short period of time to complete the rest of their development and still allow pollination to occur in the coming spring. For example, after the winter dormancy at the diffuse diplotene stage, the remaining part of meiosis in *Larix* finished within four days in the next spring (Ekberg, 1968). In slash pine (*P. elliottii*), after the winter dormancy at the pollen mother cell stage, meiosis of the pollen cone buds occurred during the middle of January; subsequent development from this stage on only took three days (Mergen, 1957). The same kind of rapid meiosis after winter dormancy was also recorded by Ferguson (1904) in *P. austriaca*, *P. strobus*, and *P. rigida*.

The first appearance of the potential pollen cone primordia of *P. radiata*, growing in New Zealand was recorded on 5/12/91 in this study, which was in the early part of the New Zealand summer. This conclusion is based on the location of these primordia on the male LSTB observed in this light microscopy study (Fig 2.4). These potential pollen cone primordia soon developed into bullet-shape primordia (Fig 2.5) and microsporophylls started to be developed on these primordia in late February (Figs 2.7, 2.8). This is when we can confidently identify these primordia as pollen cone buds. The initiation and differentiation processes of pollen cone buds started at early summer and finished in late summer. There was no apparent pause, indicating that the generally favourable temperatures in New Zealand could be a major factor responsible for this continuous development. Some slowing of later stages of pollen cone maturation may occur in the New Zealand environment, when pollen mother cells entered the prophase stage of

meiosis around late Autumn, to late May 1992. Temperatures become lower, and the subsequent meiosis process from this stage on took almost six weeks to complete before the formation of the morphologically mature pollen. This appears to contrast with the rapid development of the meiosis process in some Northern Hemisphere pine species, after their winter dormancy. This comparison may again emphasise the significant impact of the temperature factor upon "floral" development. The accelerated development of the meiosis process of the northern hemisphere pine species is an apparent result of the rapid increase of the temperature in the spring season (Mirov, 1967). In New Zealand, meiosis of pollen mother cells appears to start in late autumn, and to finish in mid-Winter. When temperatures become increasingly reduced during autumn, a slower development at the earlier stages of meiosis seems inevitable.

### 2.4.2. THE MORPHOLOGICAL ASPECTS DURING THE MALE CONE DEVELOPMENT IN *Pinus radiata*

Light microscopy not only determined the timing of some important stages of male cone development, but also revealed morphological changes that occurred during this process. When differentiation of the male cone was completed, pollen cone buds consisted of an axis and they were covered with spirally arranged microsporophylls (Figs 2.8, Fig 2.9); two microsporangia were formed on the underside of each microsporophyll. With the increase of the sporogenous cells, microsporangia became increasingly larger. In the end, microsporangia occupied the most part of the microsporophyll (Figs 2.10, 2.11. 2.15). During the later development of the male cone, the microsporophyll was much reduced and consisted of only upper epidermis (adaxial surface) and lower epidermis (abaxial surface) separated by the microsporangium (Fig 2.15). The outer walls of the upper epidermal cells were cutinised and the majority were filled with uniformly stained (apparently suberized) material. The lower epidermal cells, however, were unsuberized (Fig 2.9). This difference in the upper and lower surface tissue is most likely associated with release of the mature pollen grains caused by a dehiscence zone line along the lower epidermis. Similar events have been reported in *P. wallichiana* (Konar, 1958).

Another important feature about pollen cone development noticed in this study is that pollen cones mature at a somewhat different "rate", depending on their positions on the male cone bearing shoots. It has been reported that during "flowering" in *P. caribaea*,

growing in Northern Queensland, Australia, each cluster of pollen cones commonly released pollen at two different times; the proximal pollen cones shed first, followed two weeks later by the distal pollen cones (Harrison and Slee, 1992). This report obviously indicated that proximal pollen cones have an advanced development over the distal pollen cones. The same result was also reported by Ho and Owens (1974) who found that on a shoot of *Pinus contorta*, proximal male cones had pollen mother cells at more advanced stages than did the more distal cones. These workers also reported that the size and appearance of the male cone varied considerably during early stages of meiosis, and there appeared to be little correlation between male cone size and the stage of meiosis (Ho and Owens, 1974). Similar results were seen in this present study. Study of the changes in pollen cone size at different locations on the shoot axis from three developmental stages revealed that pollen cones located at the basal region did not have a significant difference in size compare to male cones located at the distal region (Fig 2.2). But pollen cones collected from the basal region of the pollen cone bearing shoot in early July had already showed well developed pollen grains (Fig 2.17), while pollen cones collected from the distal region of the same shoot showed only microspore tetrads (Figs 2.15, 2.16). It appears that the maturation rate of male cones relies more on their location on the shoot axis rather than on their size. This different maturation rate also applied to microsporophylls from different positions within the male cone. This difference was first noticed by Chamberlain (1935) in *P. banksiana* who presented a schematic drawing of a longitudinal section through a young male cone, and found that the microsporangia in the bottom section were in the early sporogenous stages, and that the sporogenous cells had not been differentiated in the apical part of the cone. Ho and Owens (1973) also reported that, within a male cone, pollen mother cells in the proximal microsporophylls were generally at a more advanced stage of meiosis than those in the distal part. The longitudinal section of the male cone bud collected from mid-April in this study showed a similar result. The microsporangia in the basal microsporophylls were at a much more advanced stage of development than the microsporangia in the distal microsporophylls. Sporogenous tissue had been formed in the lower section, while the sporogenous tissue cells were not very obvious in the upper section of the microsporophylls (Fig 2.9). Such a variation of male cone maturation within the shoot and within the cone itself no doubt spreads pollen release over a longer period of time, allowing a longer pollination time.

### 2.4.3. THE STRUCTURAL AND ULTRASTRUCTURAL CHANGES DURING THE MALE CONE DEVELOPMENT IN *Pinus radiata.*

Some cell structure changes during the development of pollen mother cells were noted in this study. The fate of the tapetal cells during this developmental process were also recorded. Initially, the tapetal cells appeared as an intact layer surrounding the earlier formed pollen mother cells (Fig 2.11). But this intact layer disappeared after the pollen mother cells had completed meiosis and had entered the tetrad stage, leaving only a few isolated tapetal cells along the degraded pollen-sac wall. The cells of the middle layer of the microsporangium outside the tapetal cell almost completely disappeared at this stage (Fig 2.15). In the earlier stage of the male cone, the sporogenous cells were quite compact, forming a mass of cells with no apparent intercellular spaces (Fig 2.10). However intercellular spaces became more and more obvious with the development of pollen mother cells during the meiotic process (Figs 2.11, 2.12). The nature of this free space will be discussed in relation to the observations on meiosis of the pollen mother cells using electron microscopy. Once pollen mother cells entered the prophase stage of meiosis, the signs of pairing and the definite signs of contraction of the chromosomes of the pollen mother cells were also recorded (Figs 2.13, 2.14). Similar events have also been described in other pine species. Ferguson (1904) reported that during the period preceding reduction division, the nucleus of the pollen mother cells gradually condensed, the chromosomes became thicker and the meshes of this nuclear reticulum became smaller. Contractions of chromosomes continued until the network formed a compact mass at one side of the nucleus. She pointed out that it was at the time when PMCs started reduction division (Ferguson 1904). The research of Owens and Molder (1971) on meiosis in some northern conifer species had found prolonged pachytene and diffuse diplotene stages. They observed the characteristic pachytene stage of the pollen mother cells in Douglas fir. The chromosomes appeared as thick, fuzzy strands at this stage and they were usually clumped in a tangled mass filling only a portion of the nucleus . The state of chromosomes of pollen mother cells in *Pinus radiata* collected between late May and mid-June in this study (Figs 2.13, 2.14) share similar features to chromosomes of pollen mother cells of Douglas fir at pachytene and late pachytene stages. This result

suggests that the pachytene stage of pollen mother cells in *Pinus radiata* probably started between late-May and mid-June.

Further investigation of some developmental stages of male cones were undertaken using TEM. The nature of the intercellular connections between tapetal cells and pollen mother cells, changes occurring in the cell wall of pollen mother cells during their maturation and the dedifferentiation of the cellular organelles of tapetal cells and pollen mother cells, were examined.

In the early stage of development of the microsporangium, the central sporogenous cells were slightly larger than the surrounding tapetal cells. The nuclei of cells of both tissues were found to contain two to three nucleoli. Similar observations have been reported in other *Pinus* species (Ferguson, 1904; Singh, 1978). In addition , tapetal and sporogenous cells were seen to have similar numbers of small vacuoles, and dictyosomes. Both tissues were rich in free ribosomes, mitochondria and plastids. They both had well defined cytoplasmic membranes, and the rough endoplasmic reticulum (RER) from both tissues was similar in appearance. These features, shown in Figure 2.18, are consistent with those found in cells of an actively dividing meristematic tissue.

Dickinson and Bell (1976b) also reported that early in their development the tapetal cells and sporogenous cells in *P. banksiana* were ultra-structurally alike with rich cytoplasm and having a multinucleate appearance. Some intercellular connections *via* plasmodesmata were evident at this stage both between cells of the sporogenous tissue, as well as between the tapetal cells and also between the two cell types. These features are clearly seen in Figures 2.19 and 2.20 and indicating the syncytium character of cells of both tissues, especially between the tapetal cells. This syncytium character has been described earlier in *P. banksiana* by Dickinson and Bell (1976a). They found that cytoplasmic organelles like mitochondria could even pass through the channels formed by the plasmodesmata between two tapetal cells. This remarkable observation suggests that the plasmodesmata have an extremely high size exclusion limit, much greater than is generally thought to be present between cells. The syncytium character of tapetal cells and early sporogenous cells is consistent with the probability of the two tissues developing simultaneously, and also displaying similar structural features (Fig 2.18). This similarity remained until at least the sampling time of the following month, when the callose walls around the pollen mother cells formed (Fig 2.24). Willemse (1971) made a detailed

observation on the ultra-structural changes during meiosis in *P. sylvestris*, especially on the formation of callose walls. He found that callose wall formation started at the diplotene stage of prophase I. It started from the small space between the plasma membrane and the cell wall, and the small space grew when the callose wall formation began. A fine electron dense fibrillar material accumulated against the cell wall and the flat plasma membrane, and this fibrillar network gradually changed into a highly electron transparent line between the cell wall and the flat plasma membrane. In the end, the callose wall enveloped the whole pollen mother cell and grew in thickness until the tetrad stage. From observations such as this, the transparent layer of cell walls seen at these developmental stages have been interpreted as callose walls.

Willemse's study indicated that the formation of a callose wall around pollen mother cells signals the start of meiosis. At this time pollen mother cells are isolated from their surrounding tapetal cells, and some significant ultrastructural modifications occur in both cell types. This is very different from some angiosperms species, such as *Lilium henryi* where the tapetal and sporogenous cells were distinct well before the formation of the callose wall (Dickinson and Heslop-Harrison, 1970).

Once pollen mother cells further developed towards meiosis, the tapetal cells underwent some significant structural changes. Their cell wall appeared to be thinner and autophagic vacuoles started to appear alongside the extremely dilated RER. The cytoplasm was intensely basophilic because of the density in ribosomes which seemed to be arranged into polyribosome groups (Figs, 2.31, 2.32). The relative numbers of plastids and mitochondria was reduced; they were possibly degraded by the prominent autophagic vacuoles (Figs 2.26, 2.27, 2.28, 2.29). This hypersecretory feature of the tapetum cells may allow them to secrete nutrients supplied by the tapetal cells and the mid-layer cells into the locule of the microsporangia. Subsequently these breakdown products would be expected to be taken up by the developing pollen mother cells or later on by the microspores.

The intercellular connections *via* plasmodesmata between tapetal cells and pollen mother cells and among the tapetal cells themselves were blocked by the appearance of the callosic wall among them (Figs 2.24, 2.29, 2.32), indicating that pollen mother cells and tapetal cells will develop independently along different routes: one group towards meiosis, one group differentiating towards hypersecretory tissue. A block of the interfering genetic

messages between these two tissue cells would be expected to be necessary. The callosic blockage among pollen mother cells indicated that the development of pollen mother cells from this stage on would not necessarily proceed simultaneously, because of the lack of communication among them.

Osmiophilic granules and globules were noticed on the radial surfaces of the tapetal cells and subsequently throughout the microsporangial loculus in this study (Figs 2.30, 2.35). Similar events have been recorded in *P. banksiana* (Dickinson and Bell, 1976a) and *P. sylvestris* (Rowley and Walles, 1985). The nature of these osmiophilic granules was previously investigated by gas chromatography (Brooks, 1971). Brooks found numerous carotene residues in the osmiophilic granules in sporopollenin, suggesting the lipid nature of the osmiophilic granules. Dickinson and Bell (1976a) found a clear correlation between the fall in the number of these lipid-like granules in the cytoplasm of the tapetal cell and the development of these external granules in the loculus in *P. banksiana*, and they pointed out that these granules originated in the tapetal protoplasts. They also reported that the granules were prominent in the tapetal cytoplasm at the beginning of sporopollenin production, but gradually declined as increasing amounts of sporopollenin were deposited on the pollen wall, suggesting that these lipid-like granules were possibly metabolised in the cisternae of the tapetal cell and used ultimately to form the sporopollenin on the pollen wall (Dickinson and Bell, 1976a).

In an angiosperm genus, *Raphanus*, it has been reported that pollen grains might be coated to a depth of some 5 μm with the lipid-rich sporopollenin, and it has been demonstrated that this layer plays a part in the self-incompatibility system in this plant (Dickinson and Lewis, 1973). In comparing these results with the finding of the distribution of the osmiophilic granules and globules from this present study, an assumption could be made that the *Pinus* osmiophilic granules and globules are likely to be lipid in nature, have originated from the tapetal cell and were used to form the sporopollenin on the pollen wall eventually. This assumption implies that the sporopollenin on the pollen wall has a sporophytic origin (from tapetal cells) and may be partially involved in a self-incompatibility system in *Pinus radiata*, although there is no evidence of this at present.

Mid-May was determined as the time when meiosis begins in pollen mother cells (Figs 2.11, 2.12). This determination was based on both the observations of the condensation of chromosomes by light microscopy and the occurrence of callose walls among pollen

mother cells as seen by electron microscopy. The TEM study of material from the same sampling period revealed that pollen mother cells underwent some substantial structural modifications. The cell walls appeared to be thinner, and were well coated by a thick callose wall (Fig 2.24). The cytoplasmic membrane at a later stage (mid-June) showed shrinking to some degree and formed a number of invaginations inside the callose wall apparently increasing the absorbing surface at the later pachytene stage, just before the reduction division (Figs, 2.34, 2.35). Rough endoplasmic reticulum (RER) became very dilated, more autophagic vacuoles appeared, and the density of the ribosomes, plastids and mitochondria was reduced (Figs 2.33, 2.34), compared to the earlier stages. The formation of the autophagic vesicles has been traced in differentiating cells of barley (*Hordeum sativum*) by Buvat and Robert (1979) from dictyosome vesicles and tubules that concentrate enzymes. These workers reported that some of these vesicles and tubules became autophagic vacuoles which degraded the imprisoned cytoplasmic fraction by the process of autophagy. Similar findings were also reported by Walles and Rowley (1982) in *P. sylvestris*, and by Willemse (1971) in *P. sylvestris*. The fully developed autophagic vacuoles seen here at late pachytene just before the reduction division seem likely to be responsible for the reduction of some of the free ribosomes, plastids and mitochondria.

In his review, Dickinson (1987) stated that plastids and mitochondria dedifferentiated to an almost unrecognisable state during the meiotic prophase stage in most angiosperm plant species he examined. This dedifferentiation preceded a very active period of DNA synthesis and was followed by division and dedifferentiation of most of the organelles. Recent information in angiosperm species indicates that during this developmental sequence a significant proportion of both mitochondria and plastids were degraded. In *Cosmos bipinnatus*, for example this degeneration might account for up to 20% of the total mitochondria population during prophase (Dickinson, 1986). Sheffield and Bell (1979) pointed out that meiosis in both angiosperms and gymnosperms shared some common features, including the partial dedifferentiation of the mitochondria and plastids and the appearance of vacuoles within the nuclei. The undoubted degradation of a major proportion of ribosomes during prophase in *Lilium* had been reported by Mackenzie *et al* (1967). More recently, similar events have also been found in the gymnosperm species *Taxus* (Pennell and Bell, 1986). These events have been interpreted as crucial in the reorganisation of a diploid cell (sporogenous cell) into one whose genome is haploid

(microspore), and in which expression of that part of the genome which is concerned specifically with sporophytic growth must be replaced by that part concerned specifically with gametophytes (Dickinson and Heslop-Harrison, 1977).

The transmission electron microscopy study on selected stages of the microsporogenesis in *Pinus radiata*, has generally agreed with the results achieved from other plant species and provide some ultrastructural evidence of events specifically occurring during male cone development in this gymnosperm species.

Taking the results of the light and electron microscopy studies together, the developmental events of male cone initiation and maturation of *Pinus radiata* growing in the southern hemisphere location of New Zealand have been determined, and a calendar of the annual progression of events is shown in Table **2.1**.

Table **2.1**. Some phenomena in the annual progression of events in male long shoot terminal buds of *Pinus radiata*, growing in the central north island, Rotorua, New Zealand.

| Dates | Observation |
|---|---|
| 19/11/91 | Sterile cataphylls of male shoot terminal buds initiated. Fig 2.3. |
| 5/12/91 | Short shoot initials (axially primordia) were formed. Fig 2.4. |
| 15/1 /92 | The potential bullet-shaped male cone primordia were formed. Fig 2.5. |
| 20/2 /92 | Male cone microsporophylls initiated. Fig 2.7. |
| 12/3 /92 | Developing microsporophylls were seen. Fig 2.8. |
| 19/4 /92 | Microsporangia were formed in the proximal male cone microsporophylls. Figs 2.9, 2.10. |
| 29/4 /92 | The increased number of sporogenous cells were abundant in the enlarged microsporangia.<br>Plasmodesmata were seen among sporogenous cells, tapetal cells and between them. Figs 2.19, 2.20. |
| 20/5 /92 | The tapetum was well defined, and the prophase stage of meiosis of pollen mother cells (PMCs) began.<br>Callose walls among PMCs and the condensed chromosomes of PMCs started to appear.<br>Figs 2.11, 2.12, 2.21, 2.22, 2.23, 2.24. |
| 27/5 /92 | The suggestive pairing of chromosomes were seen, PMCs were at early pachytene stage. The dedifferentiation of the subcellular organelles began in PMCs. Callosic walls among PMCs became more obvious. Figs 2.13, 2.14, 2.25, 2.26, 2.27, 2.28, 2.29. |
| 3/6/92 | Both tapetal cells and PMCs showed a hypersecretory appearance. The dilated RER system, and the cytoplasmic degradation were seen in both tissues. Osmiophilic granules appeared on the radial surface of the tapetal cells. Figs 2.30, 2.31, 2.32, 2.33. |
| 16/6/92 | Contracted chromosomes clumped in a tangled mass, showing the late pachytene stage of PMCs. The intact tapetal cell layer disappeared. A prominent invagination occurred along the cytoplasm membrane of the PMCs. Callosic walls separated PMCs from tapetal cells. The reduction of the subcellular organelles were seen at this stage. Figs 2. 34, 2.35. |
| 2/7/92 | Tapetum layer had disappeared,and microspore-tetrads were seen. Pollen release started. Figs 2.15, 2.16, 2.17. |

A comparison of developmental timetables of *Pinus radiata* growing in New Zealand with other *Pinus* species from various world locations is shown in Table **2.2**. The important events of male cone initiation and maturation of *Pinus radiata*, including microsporophyll differentiation, microsporangia formation, formation of sporogenous tissues, and the timing of the onset of meiosis are shown against a monthly time scale. Formation of pollen grains and their release is also shown. The compact and unbroken nature of this developmental sequence of events is contrasted in Table **2.2** with the interrupted sequence nature for Northern Hemisphere male cone maturation. The prominence of a dormancy period for each of four *Pinus* species from Asia and North America is clearly evident. The length of the interruption appears to reflect their geographic locations- the closer they are to the Equator, the shorter the dormancy period. The dormancy period is seen to always proceed the onset of meiosis (Table **2.2**)

A point of some debate is the timing of the onset of meiosis. General flowering developmental theory would predict that the reduction division would occur prior to the appearance of tetrads. Thus we would expect meiosis to be occurring in late June or early July. A flow cytometry study (**see appendix 1**.) was introduced to confirm this by obtaining ploidy data at selected developmental stages. Control tissues were used to set the instrumental parameters : haploid megagametophyte tissues from dissected seeds of *Pinus radiata* were used to set the 1c nuclei channel number value. The results showed that no haploid (1c) nuclei were detected at any stages - not even in the germinated pollen tubes, despite adequate number of extractable nuclei examined.

This result is puzzling and raises the question as to the timing of the actual reduction from 2c to 1c. The result from this flow cytometry study raises the probability that this event may not occur until later in pollen tube development.

Table **2.2** Comparison of developmental timetables of *Pinus* species from various world locations.

| Dates | *Pinus radiata* | *Pinus elliottii* | *Pinus banksiana* | *Pinus wallichiana* | *Pinus contorta* |
|---|---|---|---|---|---|
| | Rotorua New Zealand (Wang, 1995) | Florida, U.S.A (Mergen *et al.*,1957) | Wisconsin, U.S.A. (Curtis, *et al.*, 1972) | Eastern Himalayas. (Konar, *et al.*, 1957) | B.C. Canada. (Owens, *et al.*, 1975) |
| May | | | | | Male cones initiated. |
| June | | | | | |
| July | | Male cones initiated. | | | |
| August | | | | | |
| September | | | Male cones initiated. | | Sporogenous tissues formed. |
| October | | | **Winter dormancy began.** | | **Winter dormancy began.** |
| | | | | Male cones initiated. | |
| November | | Sporogenous tissues formed. | | **Winter dormancy began.** | |
| December | Male cones initiated. | Pollen mother cells formed. **Dormancy began.** | | **Cell division resumed.** | |
| January | | | | | |
| February | Male cones determined. | **Cell division resumed.** Meiosis began. | | Sporogenous tissues formed. | |
| March | Microsporophylls differentiated. | Pollen grains formed. | **Cell division** | Meiosis process. | |
| April | Microsporangia formed. Sporogenous tissues formed . | Pollination began. | **resumed.** Sporogenous tissues formed. Meiosis process. | | **Cell division resumed.** |
| May | Meiosis process. | | Pollination occurred. | Pollination occurred. | Meiosis process. |
| June | | | | | Pollination occurred. |
| July | Pollen grains formed. | | | | |
| August | Pollination occurred. | | | | |

# CHAPTER 3.0 MALE CONE DEVELOPMENT IN *Pinus radiata*—STUDIES OF CHANGES IN PROTEIN AND ISOENZYME PATTERNS

## 3. 1. LITERATURE REVIEW

"Developmental processes in plants, from cellular differentiation to morphological development and functional specialization, are accompanied by a continuous synthesis and degradation of specific enzymes and structural proteins. The appearance of new or increased enzyme activity in a developing organism may result from the *de novo* synthesis of the enzyme molecule or from the activation of a pre-existing enzyme precursor. Under the influence of Beadle's "one gene - one enzyme hypothesis" and the fact that there are many examples of stage and tissue-specific levels of enzyme activities during growth and differentiation of higher organisms, it has become very popular to interpret such enzyme fluctuations as the consequence of differential gene action."

As Scandalios (1974) has pointed out in the text above, studies of differential gene expression, i.e. studies of enzyme fluctuation during development, are basic to our understanding of developmental processes. One approach in such studies is to study the ontogeny of enzymes characteristic of a particular system and the appearance or disappearance of certain structural proteins, since these provide a sensitive index of basic changes occurring during differentiation.

Enzymes commonly exist in multiple molecular forms with similar or identical substrate specificity occurring within the same organism, also as known isozymes (Markert and Møller, 1959). This phenomenon presents us with markers to study the sequential development of organisms.

Multiple forms of enzymes (isozymes) might arise from gene duplication with subsequent mutations at daughter and parental loci, thus more than one gene theoretically contributes to the structure of any enzyme composed of more than one kind of subunit (Scandalios, 1974). Isozymes may also arise through the binding of a single polypeptide to varying numbers of coenzymes molecules or other prosthetic groups; or by conjugation or deletion of molecules with reactive groups such as amino, carboxyl, or hydroxyl groups of the amino acid residues of the polypeptide chain (Jacobson, 1968). Isozymes may also result from variations in the tertiary or quaternary structure of a given primary polypeptide

structure. Some isozymes may even arise during preparative procedures or during storage (Kitto *et al.*, 1966).

There are a number of techniques which have been developed to investigate enzyme distribution within plant tissue. Examples are: cytochemical methods, application of enzyme substrate to freeze-sectioned fresh tissue without prior fixation, and examination to determine whether the substrate has been utilised or not, and tissue printing onto nitrocellulose. Such tests usually utilise chromogenic substrates which change colour in the reaction. For studying changes of protein enzyme patterns, gel electrophoretic analysis methods have been successfully used.

Dunn (1989) reviewed basic principles of gel electrophoretic analysis methods. In his review, he pointed out that proteins were charged at a particular pH and thus would migrate in an electric field in a manner dependent on their charge density. If the sample was initially present in a narrow zone, proteins of different mobilities would travel as discrete zones and thus separated during electrophoresis.

A range of electrophoretic techniques are available which can separate proteins on the basis of one or a combination of their three major properties: size, net charge and relative hydrophobicity. Polyacrylamide gel electrophoresis (PAGE) has been the choice for most applications. This is because of the high resolution capacity of the polyacrylamide as a separation support medium to counteract the effects of convection and diffusion that occur during electrophoresis, and to facilitate the immobilization of the separated proteins. Electrophoresis under native conditions is often used to analyze soluble proteins with the advantage of the retention of their biological and enzymatic properties. In the presence of the anionic detergent, sodium dodecyl sulphate (SDS), proteins can be characterized by PAGE in terms of the molecular size of their constituent polypeptides under denatured conditions.

Isoelectric focusing is another gel technique of use in analytical studies. It can be performed under native conditions, by which proteins are separated in the presence of a continuous pH gradient. Under these conditions proteins migrate according to their charges until they reach the pH values (isoelectric points, pI) at which they have no charge. The proteins will attain a steady state of zero migration and will be concentrated or focused into narrow zones. The biological and enzymatic properties of proteins can be retained in this technique (Dunn, 1989).

Armed with these well-developed techniques, previous investigators have worked on protein enzymes from three different angles: (a) identifying and characterising distinct isozymes in different tissues of a given organism; (b) identifying some isozymes present in a tissue at a given developmental stage but absent at another; (c) identifying genetically identical isozymes present in different tissues but in varying quantities. For example, the development of embryos of *Zea mays* (Scandalios, 1975), leaves of *Xanthium* (Chen *et al.*, 1970), and leaves and internodes of *Populus* (Gordon, 1971) were each associated with enzyme changes in isozymes of one or more than one enzyme. The differentiation of a callus into roots, (Simola, 1973), shoots, (Mader *et al.*, 1975) and embryoids (Wochok and Burleson, 1974), was also correlated with changes in isozyme patterns.

Studies on isozyme changes during flower development, have been of interest for many researchers. Kahlem (1975) demonstrated the occurrence of specific antigens and isozymes in the inflorescence of *Mercurialis*, showing that specific peroxidase isozymes were associated with stamen development in several plant species (Kahlem, 1975). Jaiswal and Kumar (1980) reported a changing pattern of peroxidase isozymes during the development of male flowers of *Coccinia indica* (Jaiswal and Kumar, 1980). Longo reported the involvement of peroxidase isoenzymes in the sexual differentiation in *Asparagus officinalis L.* (Longo, *et al*, 1990).

A systematic enzymatic study in *Petunia hybrida* by Nave and Sawhney (1986) provided us with more detailed information about the occurrence of isozymes of esterase, peroxidase, alcohol dehydrogenase (ADH) and malate dehydrogenase (MDH) in nine stages of the post-meiotic anther development of *Petunia hybrida*. They found that the occurrence of esterase was restricted primarily to the degeneration of the tapetum . The role of esterase in the tapetum degeneration and in the hydrolysis of sporopollenin for pollen wall formation has been reported before (Ahokas, 1976, Vithange and Knox, 1976). That esterases were indeed involved in these processes had been further substantiated by the finding of lower activities of esterase in the cytoplasmic male sterile line, in which tapetal degeneration occurred early and pollen wall formation did not take place, indicating that the activity of esterase had caused or at least been associated with the early degeneration of the tapetum before the pollen wall formation took place, leading to male sterility (Hohler and Borner, 1980, Karim *et al.*, 1984, Abbott *et al.*, 1984, Bino, 1985). Nave and Sawhney's work also indicated that the activity of the ADH isozymes

were associated with anther dehiscence, while some forms of MDH were associated with all the tissues throughout the anther development. They suggested that one form of MDH might specifically be involved with the tapetum, one form with pollen maturation, and some other forms of MDH involved with the development of wall layers of the anthers. In Nave and Sawhney's study (1986), another enzyme, peroxidase was observed specifically localized in the endothecium of *P. hybrida* anthers. They pointed out that the peroxidase activity could be related to the thickening of cell walls of the endothecium

Peroxidases are known to have a role in the lignification process (Harkin and Obst, 1973), and some isozymes have been specifically related to the formation of coniferyl alcohol- a precursor of lignin (Goldberg *et al.*, 1983). In general, peroxidases are such ubiquitous enzymes that they are found in a broad range of tissue and cellular compartments, their proposed roles range from auxin catabolism (Bandurski, 1984), cross-linking of phenolic components in the wall (Fry, 1986), suberin biosynthesis (Mohan and Kolattukudy, 1990), reaction to fungal infection (Coffey and Cassidy, 1984), tracheary element differentiation (Masuda, *et al.* 1983) and they are also useful as heritable marker enzymes (Gaudreault and Tyson, 1986).

Changes in protein, peroxidase and esterase patterns during anther development in *Zea mays* was investigated by Delvallee and Dumas (1988). In their work, stages of pollen development were characterized by cytological and morphological changes and then correlated to protein and enzyme patterns. Based on significant changes of protein and enzyme patterns, they divided gametophytic development into three sequences. Detchepare *et al* (1989) also studied changes of protein patterns during anther development in *Brassica*, using SDS-PAGE. They have shown some differences in protein and glycoprotein patterns between the successive stages of pollen development in two genotypes of *B.oleracea.* Wu and Murry (1985) characterized fertile anther development versus male sterile anther development in *Petunia*, using SDS-PAGE techniques. They found that mature pollen proteins differed significantly from premeiotic pollen mother cell protein patterns in fertile lines and not in male sterile lines. They pointed out that there might be a possible block in protein synthesis in the abortive microspores. Abott *et al* (1984) showed that certain isozymes decreased in amount with age of the anther while new isoenzymes appeared during anther development in maize and they were specifically gametophytic.

Changes of enzyme activity were also noticed among a broad range of gymnosperm species. Pettitt (1977) reported that both nonspecific esterase and acid phosphatase had an intense activity between the intine and exine of the sulcus of the pollen grain in a primitive gymnosperm species, *Macrozamia redlei*, indicating that these two enzymes could be utilised in the metabolism of substrate encountered by the pollen tube during its growth through the nucellus. A similar finding was also recorded in a conifer species, *Abies alba* (Pettitt, 1985).

In extensive studies of enzyme distribution in shoot tips of white spruce (*Picea glauca*), Vanden Born (1963) reported that peroxidase was present in a number of different tissues but was associated particularly with meristematic regions. He found that peroxidase activity was high in the meristematic area at the base of young growing needles, and the base of young male cones. Peroxidase activity was observed in provascular strands, both the main longitudinal ones and the branches into the cone scale primordia, high peroxidase activity was also observed in the tapetal layer around the microsporangium. He also found that high concentrations of acid phosphatase were localized at the bases of new needle primordia and in the provascular region. In male cones, acid phosphatase was observed only at the bases of microsporophyll primordia, indicating that this enzyme was associated with the early stages of cellular differentiation (Vanden Born, 1963). Fosket and Mikshe (1966) reported a high acid phosphatase activity in the potential apical initial and central mother cell zones in *Pinus lambertiana*, but after the cells of these two regions assumed the morphological characteristics typical of the apical initial and central mother cell zones, these regions were no longer high in acid phosphatase.

From the review above it can be seen that previous studies on changes of protein and isoenzyme patterns have focused on the development and differentiation of the apical meristematic region and the development of the post-meiosis pollen grain in gymnosperm species.

The result of anatomical studies of reproductive development on *Pinus radiata* described in Chapter Two of this thesis have provided us with abundant information on male cone development at both cellular and subcellular level for this gymnosperm species. Information on possible correlations of the cytological and anatomical changes to changes of protein and isoenzyme patterns during male cone development however are rarely seen.

In this study, possible correlations between cytological differentiation stages and biochemical changes detectable in extracts taken during the course of male cone development in *Pinus radiata* were examined. To maximize the chances of finding specific patterns, three isoenzyme systems, acid phosphatase, non-specific esterase and peroxidase, which had been previously demonstrated to be highly tissue specific in their expression (Pederson, *et al*, 1987, Pederson, 1988) and another key enzyme, malate dehydrogenase, which plays a central role in many biochemical pathways within the plant cell were chosen as marker enzymes for this study. Changes of the enzyme banding patterns at different developmental stages of the male cone were studied by isoelectric focusing. Changes of total soluble protein patterns at different developmental stages of the male cone were studied by SDS-PAGE.

# 3. 2. METHODS AND MATERIALS

## 3.2.1 MATERIALS

Based on the information obtained from the anatomical study of male cone development in *Pinus radiata*, nine critical stages from the total of fourteen (see Table 2.1, in Chapter Two) have been determined, as being worthy of study to correlate stages in structural development with biochemical analysis. Male cone buds of these nine stages, fresh young needle fascicle tissue and mature pollen from clone 880-606, 880-607 and 880-612 were collected from the Forest Research Institute, Rotorua. They were stored at -20°C after they were collected.

Measurement of the length and width of male cone buds from nine developmental stages of three clonal trees was made. Ten male cone buds randomly collected from the shoot tips at each stage were measured with callipers, the average maximum length and width were taken and the standard deviation was calculated. At later stages, male cone buds from each stage were collected from shoot tips at the same length, so that the comparison was not biased. Changes of male cone bud length (bud l) and width (bud w) of nine developmental stages are described in Fig 3.1.

Protein was extracted from *Pinus radiata* tissue by a modification of the method of Mayer (1987) and analysed by SDS-PAGE and isoelectric focusing. The following sections give experimental details of the materials and methods used.

## 3.2.2 BUFFERS AND SOLUTIONS

### 3.2.2.1 Protein Extraction Buffer Modified from Mayer (Mayer, 1987)

The total soluble protein extraction buffer contained : Tris 50 mM (pH 7.5), NaC 300 mM, EDTA 1 mM, 2% Triton X-100, Ascorbic acid 5 mM and Dithiothreitol (DTT) 100 mM.

### 3.2.2.2 SDS Reducing Buffer

SDS reducing buffer (8 ml) contained : Distilled $H_20$ 4.0 ml, 0.5 M Tris 1.0 ml, Glycerol 0.8 ml, 10%SDS 1.6 ml, 2-β-mercaptoethanol 0.4 ml and 0.05% bromophenol blue 0.2 ml.

3.2.2.3 5X Electrode Buffer, PH 8.3

5 X electrode buffer (300ml) contained: 0.125 M Tris base, 7.2% Glycine, 0.5% SDS. pH was adjusted to 8.3 with 1 M NaOH/HCl. Distilled water was added to 300 ml and stored at $4^0$C.

3.2.2.4 Gel Staining Solution for SDS-PAGE

Gel staining solution was made by disolving 0.1% coomassie blue in a solution containing 40% methanol (MeOH) and 10% glacial acetic acid (HOAC).

3.2.2.5 Gel Destaining Solution for SDS-PAGE

Gel destaining solution contained 40% MeOH and 10% HOAC.

3.2.2.6 Gel Fixing Solution for Isoelectric Focusing.

250 ml gel fixing solution contained 29 g TCA (Trichloroacetic acid), 8.5 g sulphosalicylic acid. Distilled water was added to a total volume of 250 ml.

3.2.2.7 Gel Destaining Solution for Isoelectric Focusing.

Gel destaining solution contained 25% ethanol and 8% acetic acid.

3.2.2.8 Gel Staining Solution for Isoelectric Focusing.

Gel staining solution was made by dissolving 0.29 g coomassie blue in 250 ml destaining (section 3.2.2.7.) solution. The staining solution was stirred with a magnetic stirrer and heated to $60^0$C, filtered before use. The solution was used only once.

## 3. 2. 3 VISUALIZATION OF ISOZYMES

3. 2.3.1 Acid phosphatase (AC; E.C. 3.1.3.2) Staining Recipe for Isoelectric Focusing.

200 ml staining solution contained 50 ml 0.2 M sodium acetate buffer (pH 5.0), 150 mg sodium-α-naphthyl acid phosphate, 50 mg fast garnet GBC salt and 5 ml 1% $MgCl_2$ (w/v).

3.2.3.2. Non-specific esterase (EST; E.C.3.1.1) (colorimetric) Staining Recipe for Isoelectric Focusing.

200 ml staining solution contained 50 ml 0.2 M phosphate buffer (pH 6.4), 50mg each of α- and β-naphthyl acetate in 2.5ml of acetone, 100mg fast garnet GBG.

3. 2.3.3. Malate dehydrogenase (MDH; E.C.1.1.1.37) Staining Recipe for Isoelectric Focusing.

200 ml staining solution contained 12.5 ml 0.2 M Tris-HCl ( pH 8.0), 12.5 ml 0.5 M D L - malic acid (pH 7.0), 0.5 ml NAD (Nicotinamide Adenine Dinucleotide) ( 10mg/ml), 0.5 ml MTT (3-[4,5-Dimethylthiazol-2-yi]-2,5-diphenyltetrazolium bromide) (10mg/ml) and 0.5 ml PMS (Phenazine Methosulfate) (2mg/ml).

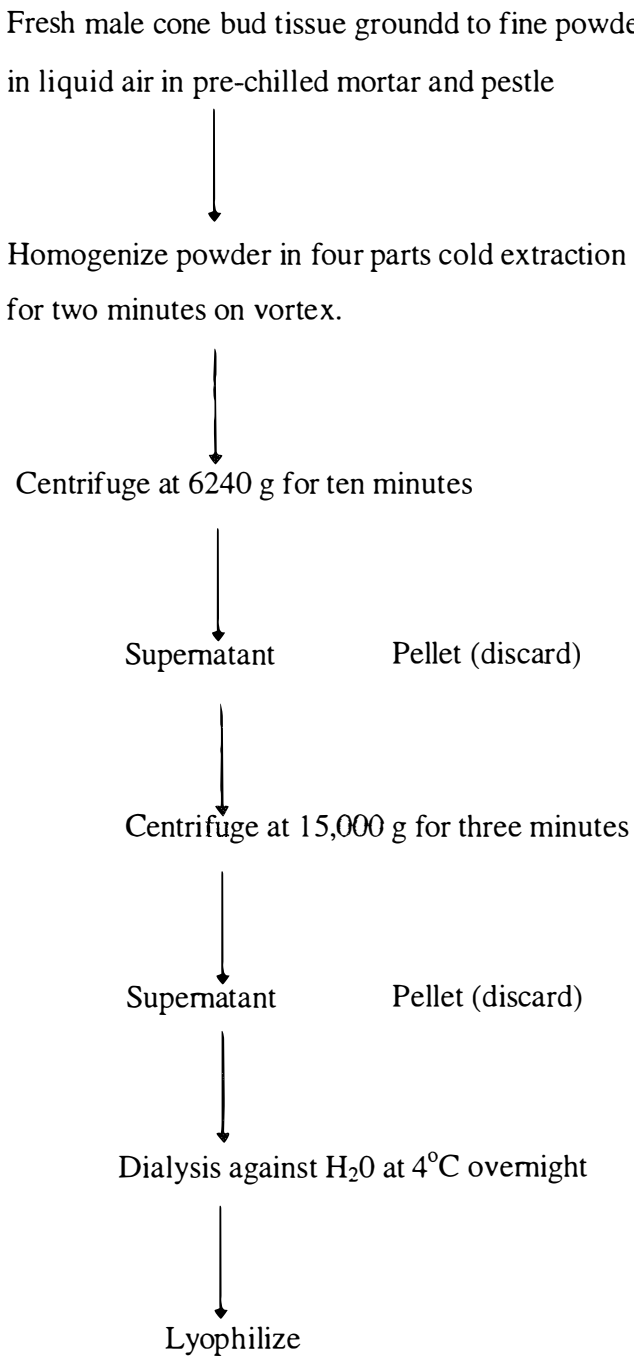
3. 2.3.4. Peroxidase (PRX; E.C.1.11.1.7) Staining Recipe for Isoelectric Focusing.

200 ml staining solution contained 50 ml 50 mM sodium-acetate buffer (pH5.0), 50mg $CaCl_2$, 1ml 3% hydrogen peroxide and 50 mg 3-amino-9-ethylcarbazole dissolved in 5ml N,N-dimethylformamide solution.

## 3. 2.4. PROTEIN EXTRACTION AND QUANTIFICATION

3. 2.4.1. Protein Extraction.

Male cone buds were dissected under the dissecting microscope. The sclerified bracts were taken off. Only the fresh cone buds were retained. An average of 2.5 grams fresh male cone bud tissues and needle fasicle tissues were used for protein extraction. The protein extraction protocol based on methods developed by Butcher *et al* (1981) modified from Mayer (1987) was shown in the following diagram:




The soluble protein sample was dissolved in 500µl Milli Q $H_20$, and stored at -20°C for protein quantification, SDS-PAGE analysis and isoelectric focusing.

The protein extraction buffer (section 3. 2.2.1) was modified from Mayer (1987) which was developed for use with a wide variety of plant species including gymnosperms.

3. 2.4.2. Protein Quantification.

Protein quantification used the method of Bradford (Bradford, 1976).

## 3. 2. 5. SDS - PAGE

3. 2.5.1. Protein Sample and Gel Apparatus Preparation for SDS-PAGE

The protein sample solution was diluted with four times the volume of the SDS reducing buffer (section 3.2 2.2). Standard marker proteins were used to assess molecular weight of bands. The markers used were [Bio-Rad SDS-PAGE low molecular weight standards: Lysozyme (hen egg white) 14.4 kilodaltons (KD), Soybean trypsin inhibitor 21.5 KD, Carbonic anhydrase (bovine) 31 KD, Ovalbumin (hen egg white) 42.69 KD, Bovine serum albumin 66.2 KD, Phosphorylase B (rabbit muscle) 97.4 KD and β-galactosidase (*E.Coli*) 116.25 KD]. The standard marker protein was diluted with twenty times the volume of the SDS reducing buffer (section 3.2.2.2). The mixture was boiled for five minutes and then cooled on ice. Samples and standard proteins were then ready to load on to the gel. Vertical slab SDS-PAGE was carried out using the discontinuous buffer system described by Laemmli (Laemmli, 1970). Polyacrylamide gels (stacking gel 4%; separating gel 12%) were prepared and run in a Bio-Rad mini-protein II slab cell in a 5 X electrode buffer (section 3.2.2.3.) Equivalent amounts of protein per well were loaded for each sample (20 μg for total protein staining). Gels were prepared according to the following protocols:

3. 2.5.2. Gel Preparation for SDS-PAGE

Separation gel: 12%

| | |
|---|---|
| Distilled $H_2O$ | 3.35 ml |
| 1.5 M Tris-HCl (PH 8.8) | 2.5 ml |
| 10% SDS | 1.0 ml |
| Acrylamide-Bis | 4.0 ml |
| (Acrylamide/Bis: 37.5:1) | |
| (de-gas at room temperature for 15 minutes) | |
| 10% Ammonium persulfate | 50 μl |
| (freshly made) | |
| Temed | 5 μl |
| (N,N,N',N'-tetramethylethylenediamine) | |

Stacking gel: 4%

| | |
|---|---|
| Distilled $H_2O$ | 3.98 ml |
| 0.5 M Tris-Hcl (PH 6.8) | 1.58 ml |
| 10% SDS | 62.5 µl |
| Acrylamide-bis | 625 µl |
| (Acrylamide/Bis : 37.5:1) | |
| (de-gas at room temperature for 15 minutes) | |
| 10% Ammonium persulfate | 31.25 µl |
| (freshly made) | |
| Temed | 6.25 µl |
| (N,N,N',N'-tetramethylethylenediamine) | |

3. 2.5.3. Gel Running Condition for SDS-PAGE

Gels were run at a constant voltage of 200 V for 45 minutes each time.

3. 2.5.4. Gel Staining and Destaining for SDS-PAGE

Gels were stained with coomassie blue solution (section 3.2.2.4) for 30 minutes and then transferred into destain solution (section 3. 2.2.5) for 1-3 hours.

3. 2.5.5. Silver Staining for SDS-PAGE Gels

After the gels were stained with coomassie blue, they were destained for 60 minutes in a destaining solution (section 3.2.2.5). Destained gels were first immersed two times into a solution containing 10% ethanol (ETOH) and 5% HOAC, each time allowing 15 minutes, then transferred into a 200 ml solution containing 10% oxidiser (Bio-Rad) washed with distilled water for two times (each time allowing 5 minutes) and then stained in a silver reagent (Bio-Rad) (20 ml silver reagent concentrate + 180 ml Milli Q water). Gels were rinsed with Milli Q water for 30 seconds and examined in a developing solution (6.4 gram developer powder dissolved into 400 ml Milli Q water) (Bio-Rad). The developing reaction was stopped by transferring gels into a solution containing 10 ml HOAC and 190 ml Milli Q water.

## 3. 2.6 ISOELECTRIC FOCUSING GEL ELECTROPHORESIS:

### 3. 2.6.1. Protein Sample and IEF Gel Preparation

To characterize the isoenzyme activity, Ampholine PAGplates were used for analytical isoelectric focusing gel electrophoresis under native conditions (Pharmacia - LKB, 1990). The Ampholine PAGplate is a 1mm thick polyacrylamide gel incorporated with the low molecular weight carrier ampholyte cast on a plastic support film. A broad pH range (pH 3.5-9.5) Ampholine PAGplate was used for this study.

Extracted protein samples were thawed and centrifuged using a Beckman TL-100 Ultracentrifuge at 218, 000 g for one hour at 4°C to remove insoluble materials.

The Multiphor II electrophoresis unit was connected to the multi-temp II thermostatic circulator at $4^0$C 20 minutes before starting the analysis. One third of the gel was used at a time, the gel was cut into three parts, the parts to be saved were sealed with tape and stored in a refrigerator. The transparent film and aluminium foil and the thin transparent plastic film protecting the gel were removed carefully.

About 1 ml of insulating fluid (kerosene) was pipetted onto the cooling plate of the Multiphor II and the gel was positioned on the plate using the screen print as a guide, making sure no air bubbles were trapped beneath the gel.

The electrode strips were evenly soaked with 1 M $H_3PO_4$ at the anode, and with 1M NaOH at the cathode. The electrode strips were applied to the long edges of the gel.

### 3. 2.6.2. Running Condition for the IEF gel

The gel was prefocused for 15 minutes before samples were loaded. The IEF gel was run at a voltage of 1500 V, power of 15 W and current of 250 mA.

### 3. 2.6.3. Sample Application for the IEF Gel

Samples were applied 10mm from the cathode, equivalent amounts of protein per well were loaded for each sample. (the average amount of protein was 20 μg).

Sample solution was pipetted onto a stacked sample of application pieces. (filter paper trimmed into small square pieces).

After prefocussing for 15 minutes, the Multiphor II unit was switched on and the gel was run at 4°C for 90 minutes.

3. 2.6.4 Determination of the Isoelectric Point

Marker proteins of known pI were run parallel with unknown samples on the isoelectric focusing gel. After focusing, the marker protein lane was cut away from the gel and stained with coomassie staining solutions (section 3.2.2.8.). The migration distance from the cathodic edge of the gel to the different marker protein bands were plotted on the Y-axis and the corresponding pIs of the marker proteins were plotted on the X-axis. A calibration curve was drawn. By measuring the migration distance of the unknown protein, the isoelectric point of the unknown protein enzyme was interpolated.

3. 2.6.5. Staining and Destaining of the Isoelectric Focusing Gel

Immediately after isoelectric focusing, the gel was placed in the Multiphor staining Kit containing 250 ml fixing solution (section 3.2.2.6.) and left for 0.5-1 hour. This solution precipitates the proteins and allows the carrier ampholyte to diffuse out of the gel. The gel was washed once in destaining solution (section 3.2.2.7.) for 5 minutes and then stained in staining solution (coomassie blue solution) (section 3.2.2.8.) for 10 minutes. The staining solution was preheated to $60^0$C before use. The IEF gel was destained by changing the destaining solution (section 3.2.2.7.) several times until the background was clear. This procedure was also designed to examine the integrety of the total soluble protein extracted from pine tissue.

3. 2.6.6. Methods of visualization of isozymes on IEF Gels adapted from Cheliak and Pitel (1984)

After the marker protein lane was cut away, the rest of the gel was immersed into four different enzyme staining solutions respectively. For acid phosphatase: (AC; E.C. 3.1.3.2), the gel was immersed in a staining solution described in section 3. 2.3.1., and incubated for 90 minutes in the dark at room temperature until brown-black bands appeared. For non-specific esterase (EST; E.C.3.1.1), the gel was immersed in a staining solution described in section 3. 2.3.2., and incubated at room temperature for 60 minutes until red-brown bands appear. For malate dehydrogenase (MDH; E.C.1.1.1.37), the gel was immersed in a staining solution described in section 3. 2.3.3., and incubated in the dark at room temperature for 45 minutes until dark blue bands appeared. For peroxidase (PRX; E.C.1.11.1.7), the gel was stained in a solution described in section 3. 2.3.4., and incubated at room temperature for two hours until red bands appeared.

Each enzyme assay has been repreated for three times, and they all showed same result.

# 3. 3. RESULTS

## 3.3.1 SELECTION OF DEVELOPMENTAL STAGES OF MALE CONES IN *Pinus radiata* FOR ANALYSIS AT THE PROTEIN LEVEL.

From the anatomical study described in Chapter Two, nine developmental stages of the male cone were identified as components of the continuum of the developmental programme. These nine stages are shown in Table **3.1**.

Table **3.1** Cytological characterization of the nine developmental stages of the male cone of *Pinus radiata.*

| Stages | Cytological characterization |
|---|---|
| **I.** | Male cone buds were covered by newly initiated microsporophyll primordia, indicating the starting point of visible male cone development. |
| **II.** | Sporogenous tissue laid down in the basal microsporophylls of male cone buds. |
| **III.** | Microsporangia were well defined in all the microsporophylls of male cone buds. |
| **IV.** | Maximal number of sporogenous cells had filled up the microsporangium, enlarged microsporangia occupied most part of the microsporophyll. |
| **V.** | Sporogenous cells differentiated into pollen mother cells. The meiosis of pollen mother cells (PMCs) had just started. The intact tapetum layer was well defined. |
| **VI.** | PMCs of male cone buds were at the early pachytene stage of meiosis. Dedifferentiation of subcellular organelles began in PMCs. |
| **VII.** | PMCs of male cone buds were at the mid-pachytene stage of meiosis. Both tapetal cells and PMCs showed a hypersecretory appearance. The dilated RER (rough endoplasmic reticulum) system and cytoplasmic degradation were seen in both tissues. |
| **VIII.** | PMCs of male cone buds were at the late pachytene stage of meiosis, just prior to the two continuous reduction division. A prominent invagination occurred along the cytoplasmic membrane of the PMCs. The reduction of the subcellular organelles was seen at this stage. The intact tapetal cell layer disappeared |
| **IX.** | Microspore-tetrad and pollen grains were seen in microsporangia in male cone buds. |

The developmental stages were approximately related to the length and width of male cone buds (Fig 3.1). There was a significant increase in length and width of male cone buds from stage I. to stage II. Bud length increased from around 3.5 mm to around 4.5 mm. Bud width increased from below 2 mm to around 2.5 mm. From stage II, bud width had a more steady increase than the bud length, until it reached its maximum width at around 3.3 mm at stage IX. While bud length showed some variation at later stages, the general trend was to increase until it reached its maximum length at around 6 mm at stage IX. As the microsporophylls of male cone buds differentiated and developed acropetally and each microsporophyll grew outwards during its maturation, the increase of the male cone bud width was directly caused by the development of microsporophylls, so changes of the bud width were more closely related to the reproductive growth than the bud length. Bud length was more closely related to the activity of the male cone bud apex, especially at the early stages when only the basal microsporophylls differentiated.

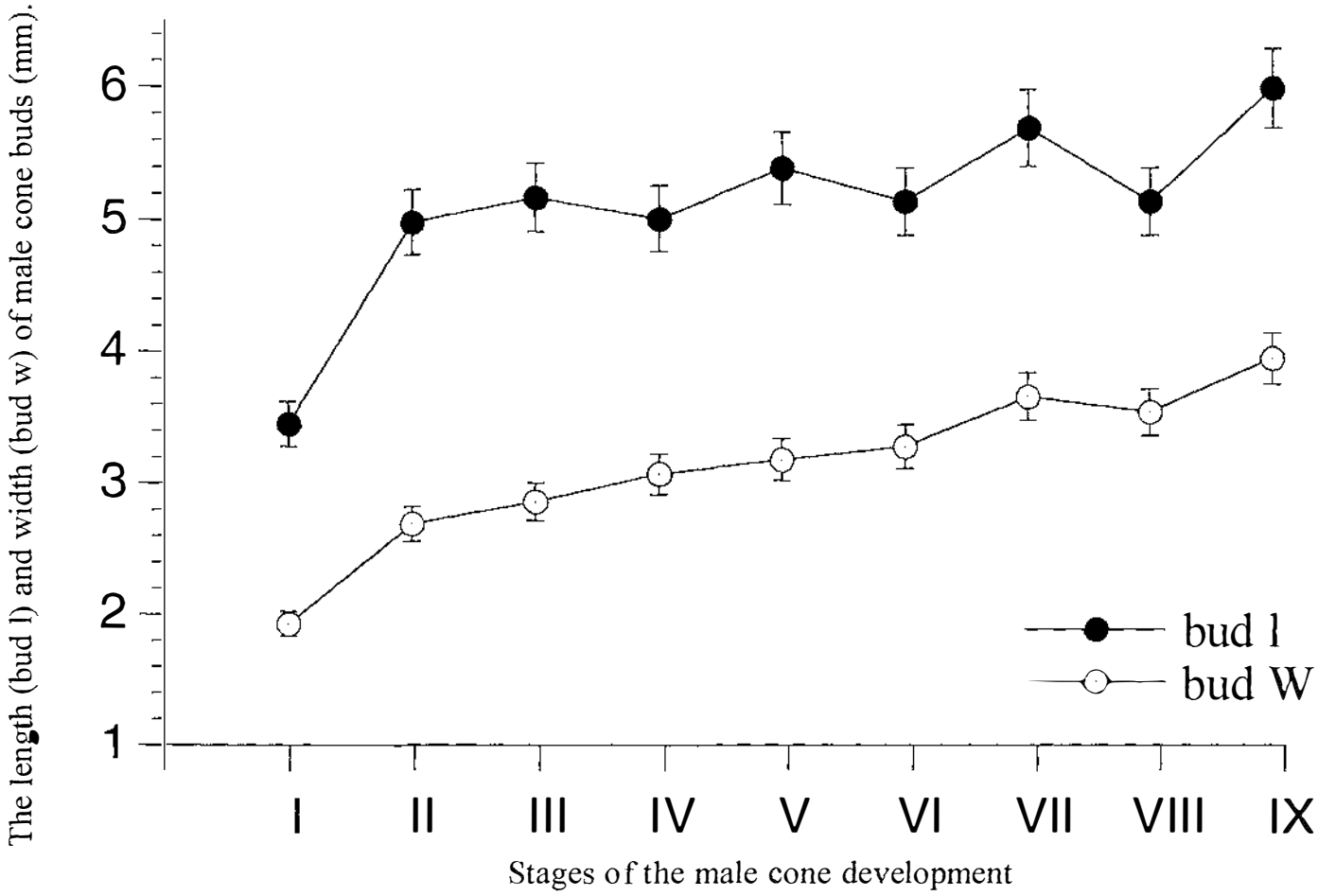



Fig 3.1 Changes of length and width of male cone buds randomly collected from pine shoots of nine stages(I-IX). Each value is the mean of ten samples and the bars indicate the standard deviation of the mean.

### 3.3.2 SOLUBLE PROTEIN CONTENT OF MALE CONE BUDS FROM EIGHT DEVELOPMENTAL STAGES.

Changes in total soluble protein content, expressed on a per gram dry tissue basis was evident, at eight critical developmental stages when protein quantification at each stage was made. The result is shown in Fig 3.2. As the major part of male cones of stage IX were covered with mature pollen sacs full of pollen grains, the protein quantification of the male cone of stage IX was omitted. For SDS-PAGE and isoelectric focusing, proteins were extracted from pollen grains instead of the male cone of stage IX.

At stage I the extractable total soluble protein content of tissue was at its highest, microsporophylls had just initiated around the male cone bud, but there were no signs of any sporogenous tissue differentiated in the microsporophyll, i.e. there were no signs of reproductive initials. The protein content here was at its highest peak, compared with the rest of the developmental stages of male cone buds. When male cone buds reached stage II, well defined microsporophylls were formed and microsporangia were also formed in the basal microsporophylls, i.e. reproductive initials had occurred, but the protein content showed a sharp drop, and it decreased to its lowest level at stage VII, when pollen mother cells in microsporophyll of the male cone were proceeding into the late pachytene stage of meiosis.

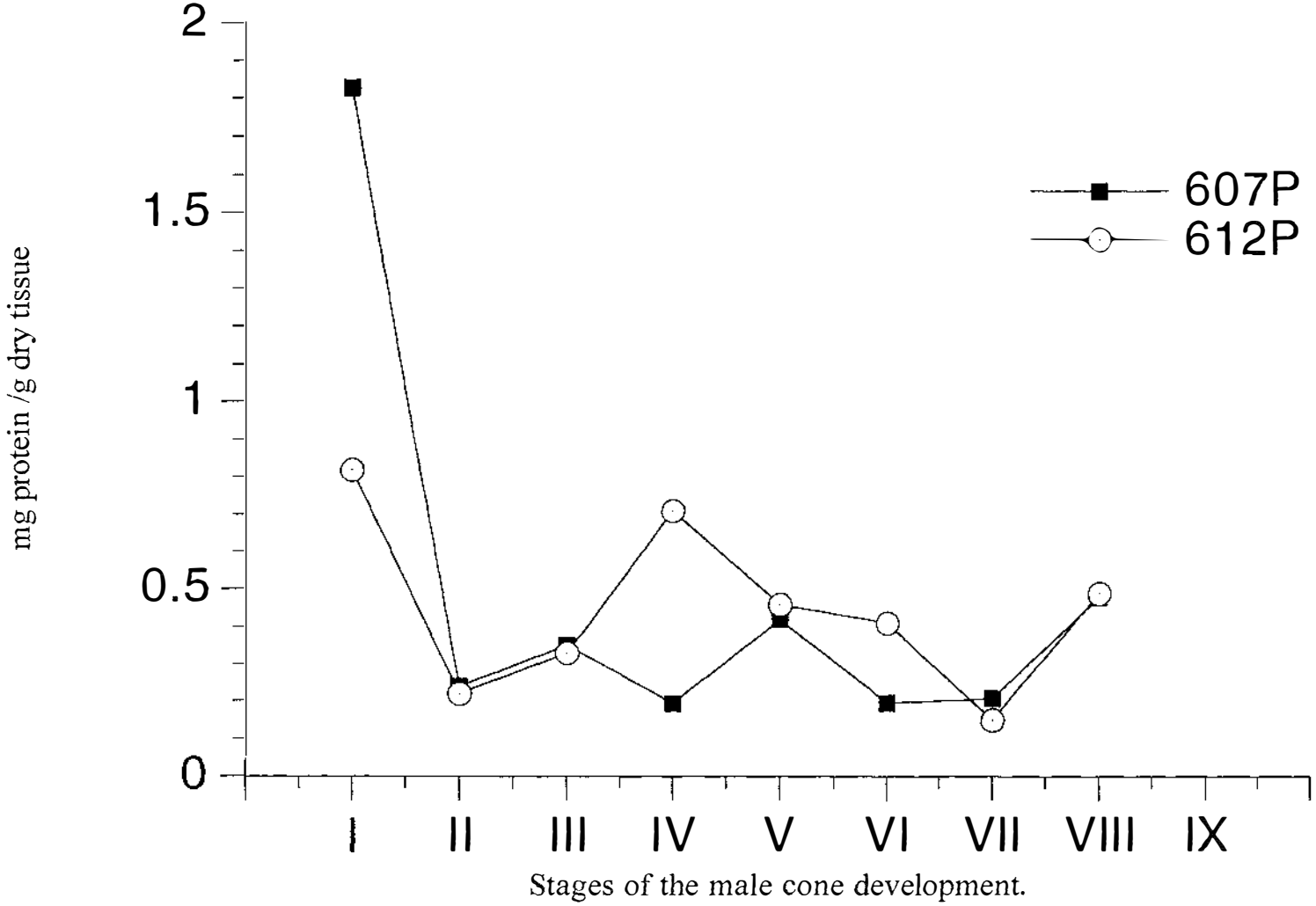



Fig 3.2 Changes in soluble protein content of radiata pine clone 880-607 (607P) and 880-612 (612P) at eight developmental stages.

## 3. 3.3 CHANGES IN SDS-PAGE PROTEIN PATTERNS DURING MALE CONE DEVELOPMENT.

Soluble proteins from different developmental stages were separated on SDS-PAGE gels and stained with coomassie blue (section 3. 2.2.4.). After destaining (section 3. 2.2.5.), the gels were subsequently stained with silver reagent (section 3. 2.5.5.) to reveal the finer changes of the protein banding patterns (Fig 3.3).

Changes of the protein banding patterns are analyzed in Table **3. 2**.

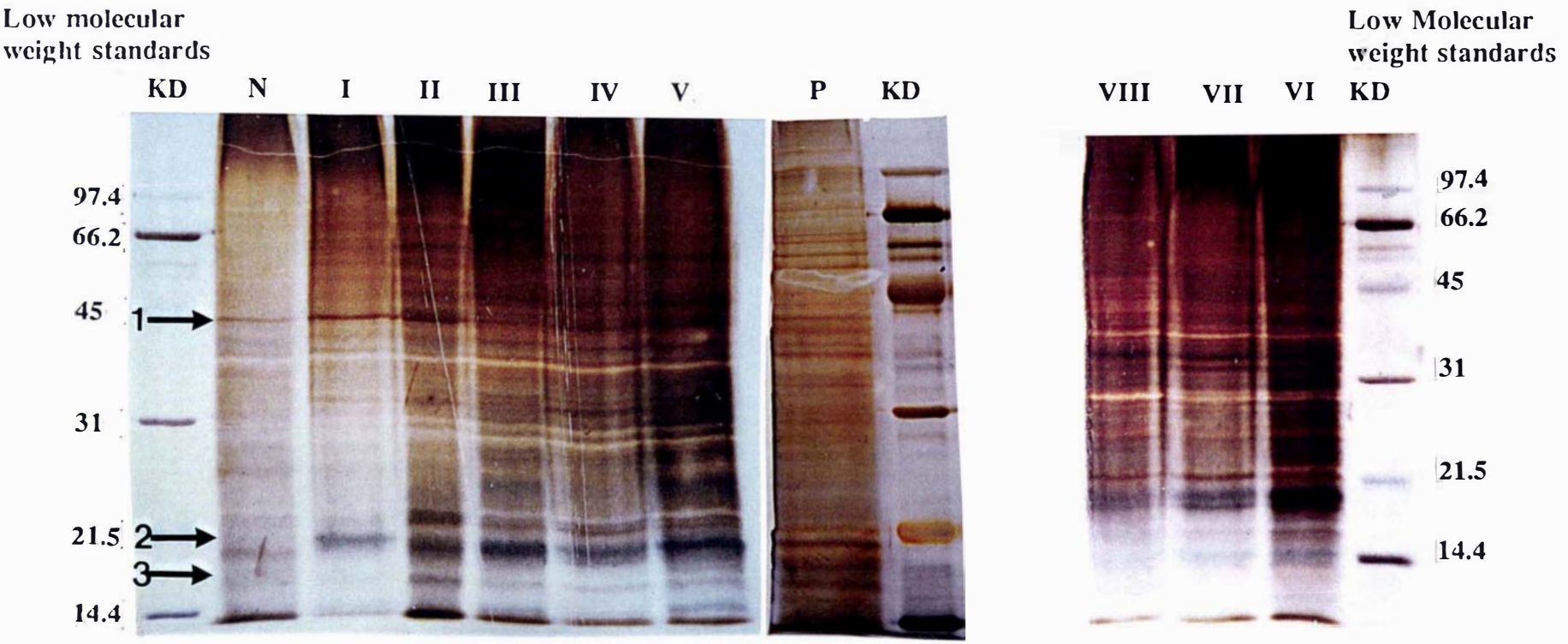

Low molecular
weight standards
KD
N
I
II
III
IV
V
P
KD
97.4
66.2
45
1
31
21.5
2
3
14.4
Low Molecular
weight standards
VIII
VII
VI
KD
97.4
66.2
45
31
21.5
14.4

Table **3.2.** SDS-PAGE study of changes of protein banding patterns during male cone development.

| protein species (KD) | N | I | II | III | IV | V | VI | VII | VIII | P |
|---|---|---|---|---|---|---|---|---|---|---|
| 97.4 | | | | | | | | | | + |
| 71.5 | | | | | | | | | | + |
| 66.2 | | | | | | | | | | + |
| 61.5 | | .. | + | + | + | + | + | + | .. | ++ |
| 59.5 | | | | | | | | | | ++ |
| 55.4 | | | | | | | | | | ++ |
| 53 | | .. | + | + | + | + | + | + | .. | ++ |
| 43.4 | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + | |
| 41.8 | + | + | + | + | + | + | + | + | + | ++ |
| 38.8 | + | + | + | + | + | + | + | + | + | ++ |
| 36.5 | + | | .. | .. | .. | .. | .. | .. | .. | .. |
| 33.4 | | .. | + | .. | + | + | + | + | + | + |
| 31.5 | | .. | + | + | + | + | + | + | + | + |
| 30.5 | .. | .. | + | + | + | + | + | + | + | + |
| 28.5 | | | | + | + | + | + | + | + | + |
| 27.8 | | | | + | + | + | + | + | + | + |
| 26 | + | | | + | | + | + | + | + | + |
| 22.5 | | | + | + | + | + | | | | |
| 22 | + | + | + | + | + | + | + | + | + | + |
| 20.5 | | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 19.5 | + | .. | + | + | ++ | ++ | ++ | ++ | ++ | + |
| 17.4 | .. | .. | + | + | + | + | + | .. | .. | ++ |

++: intense bands, +: evident bands, ..: bands with weak intensity
N: needle fascicle tissue, P: pollen
I-VIII: eight developmental stages of the male cone.

Soluble proteins extracted from mature pollen revealed more protein species than those extracted from any other earlier stages of the male cone buds, with sizes ranging from 17.4 KD to 97.4 KD. Protein species of 43.4 KD, 41.8 KD, 38.8 KD and 22 KD, were present at all stages, from vegetative needle fascicle tissue to mature pollen. The protein species of 43.4 KD (arrow 1, Fig 3.3) especially showed a stronger intensity in vegetative tissue and earlier male cone bud tissue than the later more developed male cone bud tissues. Protein species at 19.5 KD and 30.5 KD existed at all stages, but their bands showed a stronger intensity at later stages of the male cone bud tissue. Protein species of 33.4 KD; 31.5 KD and 20.5 KD only showed their existence in the male cone bud tissue and mature pollen, but were not evident in the vegetative needle fascicle tissue. A 20.5 KD protein in particular (arrow 2, Fig 3.3) showed a strong intensity band pattern at all male cone bud stages including pollen but was not seen in vegetative tissue, indicating that it could be a reproductive tissue specific protein species.

Protein species of 27.8 KD and 28.5 KD only existed in the later developmental stages of male cone buds and pollen, indicating that they could be more closely related to the meiosis of pollen mother cells and pollen development. One protein species of 22.5 KD showed a strong appearance at the male cone tissue of stage II, III, IV and V, but disappeared in the later stages of the male cone tissues, indicating its apparently exclusive involvement in the early developmental stages of the male cone, and possible involvement in the development of the microsporangia. A protein species of 17.4 KD showed a very weak band in the vegetative needle tissue and stage I male cone tissue, but showed a strong band at stage II male cone tissue and then gradually decreased its intensity to almost disappearence at stage VIII, a band of similar molecular weight was present in pollen extracts (arrow 3, Fig 3.3).

## 3.3.4 . CHANGES IN ISOENZYME PATTERNS DURING MALE CONE DEVELOPMENT

### 3. 3.4.1. Study of the Enzyme Activity of Acid Phosphatase by Isoelectric Focusing

The enzyme activity of acid phosphatase studied using native IEF gel methodology is shown in Fig 3. 4. Isoform changes were analyzed and are presented in Table **3.3.**

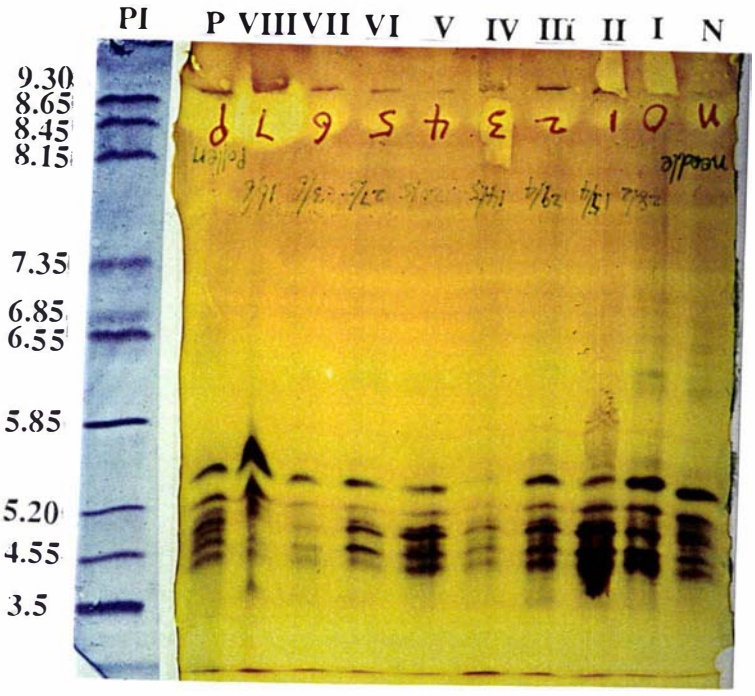



Fig 3.4. Isoelectric focusing gel electrophoresis study of the enzyme activity of acid phosphatase during male cone development in *Pinus radiata*. pI values were determined as described in the text (section 3. 2.6.4.). The numbers along the top of the figure refer to developmental stages described in Table **3.1**. N is needle tissue, P is an extract of mature pollen. The enzyme activity is expressed as brown-black bands. See text for further detailed description of the gel.

Table **3. 3**. Isoelectric focusing study of changes of the enzyme activity of acid phosphatase during male cone development.

| enzyme isoforms (pI) | N | I | II | III | IV | V | VI | VII | VIII | P |
|---|---|---|---|---|---|---|---|---|---|---|
| 3.95 | | | ++ | | | | | | + | |
| 4.40 | ++ | ++ | ++ | ++ | + | ++ | + | + | | + |
| 4.50 | | | | | | | | | + | |
| 4.60 | ++ | ++ | ++ | ++ | + | ++ | ++ | + | | ++ |
| 4.75 | | | | | | | | | + | |
| 4.85 | | | | | | | | | | + |
| 4.95 | ++ | ++ | ++ | ++ | + | ++ | ++ | + | | + |
| 5 | | | + | + | .. | + | + | | | + |
| 5.20 | | | | | | | | | + | + |
| 5.25 | ++ | ++ | ++ | + | | + | + | + | + | + |
| 5.30 | | | | | | | | | ++ | |
| 5.50 | ++ | ++ | ++ | ++ | | ++ | ++ | ++ | | ++ |
| 5.60 | | | | | | | | | ++ | |

++: intense bands, +: evident bands, ..: bands with weak intensity.
N: needle fascicle tissue, P: pollen
I-VIII: eight developmental stages of the male cone.

Thirteen isoforms of this enzyme were revealed from the vegetative needle fascicle tissue, pollen extracts and male cone tissue of eight developmental stages, with pI ranging from pH 3.95 to pH 5.60. Three isoforms with pI 4.4; 4.6 and 4.95 were consistently present from all tissues, except the male cone tissue of stage VIII. An isoform with pI 5.25 existed in all tissues, but not from the male cone tissue of stage IV, while the isoform with pI 5.5. appeared in all tissue, but not from the male cone tissue of stage IV and stage VIII. There were five dominant isoforms consistently present in most tissues, but their intensity was relatively strong in the early stages of the male cone development. Enzyme extracted from the male cone tissue of stage II showed a very strong intensity. Seven isoforms were all intensely stained. One isoform with pI 3.95 showed its strongest appearance at this stage and one isoform with pI 5 started to occur from this stage and maintained its appearance throughout stages III, IV, V, and VI, but disappeared from stage VII on. Enzyme extractable from the male cone tissues of stage VIII showed a different pattern. Two strong intensely stained bands with pI 5.60 and 5.30 occurred, which were unique to the male cone tissue of this stage. Two other isoforms with pI 4.5 and 4.75 which were not so intensely stained were also unique to the tissue of this stage. Seven isoforms occurred at stage VIII, similar to stage II.

For protein extracted from pollen, apart from the five dominant isoforms existing in this tissue, an isoform with pI 4.85 was unique to pollen extract, while an isoform with pI 5.20 only existed in pollen extract and male cone tissue of stage VIII.

3. 3.4.2. Study of the Enzyme Activity of Non-specific Esterase by Isoelectric Focusing

The enzyme activity of non-specific esterase was studied using the method described in section 3.2.6. The result is shown in Fig 3.5. Isoform changes were analyzed and are presented in Table **3. 4**.

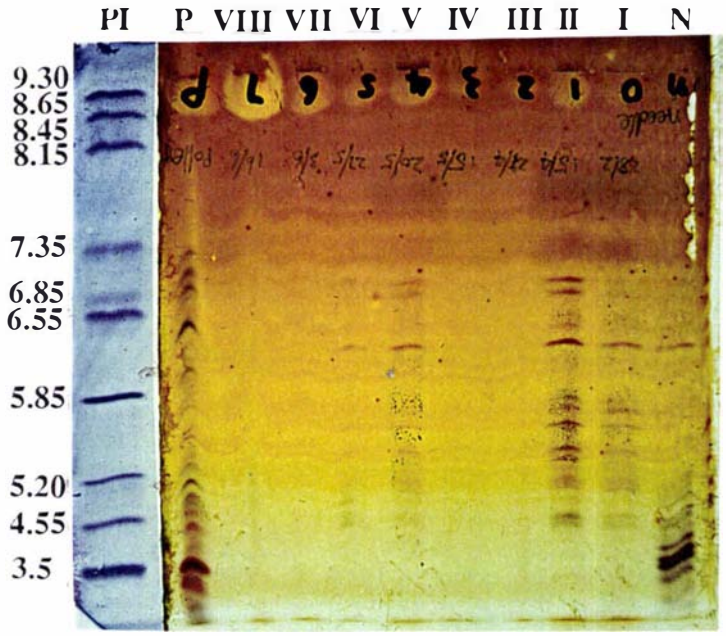



Fig 3.5. Isoelectric focusing gel electrophoresis study of the enzyme activity of non-specific esterase during male cone development in *Pinus radiata.* pI values were determined as described in the text (section 3. 2.6.4.). The numbers along the top of the figure refer to developmental stages described in Table **3.1**. N is needle tissue, P is an extract of mature pollen. The enzyme activity is expressed as red-brown bands. See text for further detailed description of the gel.

Table **3.4.** Isoelectric focusing study of changes of the enzyme activity of non-specific esterase during male cone development.

| enzyme isoforms (pI) | N | I | II | III | IV | V | VI | VII | VIII | P |
|---|---|---|---|---|---|---|---|---|---|---|
| 3.3 | ++ | | | | | | | | | ++ |
| 3.6 | ++ | | | | | | | | | ++ |
| 3.75 | + | | | | | | | | | |
| 4.2 | + | | | | | | | | | |
| 4.4 | .. | .. | .. | | | | | | | + |
| 4.65 | .. | .. | .. | | | | | | | |
| 4.7 | .. | | | | | | | | | + |
| 4.85 | .. | | | | | | | | | + |
| 4.95 | .. | .. | .. | | | | | | | + |
| 5 | .. | .. | .. | | | .. | | | | .. |
| 5.05 | .. | | | | | | | | | |
| 5.2 | | | + | | | | | | | |
| 5.3 | | .. | + | | | .. | | | | .. |
| 5.55 | | + | + | | | .. | | | | |
| 5.75 | | + | + | | | | | | | |
| 5.9 | | | .. | | | | | | | |
| 6.05 | | | | | | | | | | + |
| 6.4 | + | + | + | | | + | | | | + |
| 6.55 | | | .. | | | | | | | |
| 6.75 | | | .. | | | | | | | |
| 6.85 | | | + | | | + | | | | + |
| 7 | | | + | | | | | | | + |
| 7.25 | | | | | | | | | | + |

++: intense bands, +: evident bands, ..: bands with weak intensity.
N: needle fascicle tissue, P: pollen.
I-VIII: eight developmental stages of the male cone.

Twenty three isoforms of this enzyme were found throughout the male cone development, but most isoforms appeared in the pollen extracts, male cone tissues in early stages and vegetative tissue. Isoforms with pI 3.3 and 3.6 showed strong intensity only in the vegetative tissue and pollen extracts. Isoforms with pI 3.75; 4.2 and 5.05 existed only in the vegetative tissue. Isoforms with pI 4.4 and 4.95 showed weak appearance in vegetative tissue and male cone tissue of stage I and stage II, disappeared at later stages, but were present in pollen extracts. An isoform with pI 4.65 had only a weak appearance in the vegetative tissue; stage I and stage II, but never reappeared again at later stages. Isoforms with pI 4.7 and 4.85 showed a weak appearance in the vegetative tissue but reappeared strongly in the pollen extracts. An isoform with pI 5.2 with an intense stained band as well as isoforms with pI 5.9; 6.55 and 6.75 with less intensely stained bands seemed only to exist in stage II, but not in any other stages of the pine tissue. Isoforms with pI 5.3; 5.55 and 5.75 showed stronger appearance at stage I and II, with a weaker appearance at stage IV and pollen extracts. Isoforms with pI 6.85 and pI 7 appeared at stage II and stage V, and pollen extracts, indicating that these isoforms of the enzyme may only be expressed or function at selective stages during male cone development. Isoforms with pI 6.05 and pI 7.25 were unique to pollen extracts. There were no signs of the enzyme activity from the male cone tissue at stages III, IV, VI and VII.

3.3.4.3. Study of the Enzyme Activity of Malate Dehydrogenase by Isoelectric Focusing

The enzyme activity of malate dehydrogenase during the male cone development was studied (Section 3. 2.6.) and is revealed in Fig 3.6. Analysis of the result is presented in Table **3.5**.

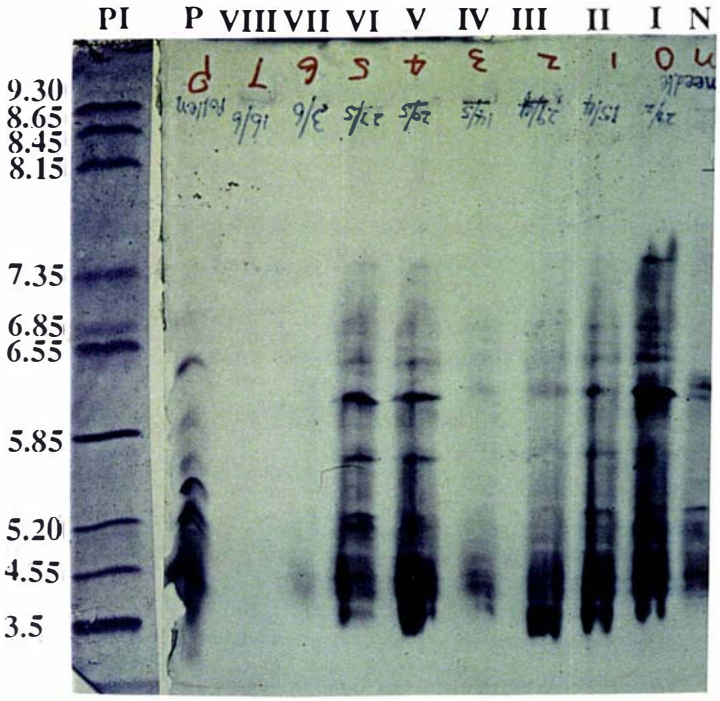



Fig 3.6. Isoelectric focusing gel electrophoresis study of the enzyme activity of malate dehydrogenase during male cone development in *Pinus radiata*. pI values were determined as described in the text (section 3.2.6.4). The numbers along the top of the figure refer to developmental stages described in Table **3.1**. N is needle tissue, P is an extract of mature pollen. The enzyme activity is expressed as dark blue bands. See text for further detailed description of the gel.

Table **3.5**. Isoelectric focusing study of changes of the enzyme activity of malate dehydrogenase during male cone development.

| enzyme isoforms (pI) | N | I | II | III | IV | V | VI | VII | VIII | P |
|---|---|---|---|---|---|---|---|---|---|---|
| 3.75 | | | ++ | ++ | .. | + | + | | | + |
| 4.25 | + | ++ | ++ | ++ | + | ++ | ++ | | | ++ |
| 4.55 | + | ++ | ++ | ++ | + | ++ | ++ | | | ++ |
| 5 | | ++ | + | ++ | + | ++ | ++ | | | ++ |
| 5.05 | + | ++ | + | .. | | + | + | | | + |
| 5.15 | + | ++ | | | | + | + | | | + |
| 5.30 | | ++ | + | | | + | + | | | ++ |
| 5.40 | | | | | | | | | | ++ |
| 5.60 | | | | | | | | | | ++ |
| 5.70 | | ++ | | | | | | | | |
| 5.80 | | ++ | + | .. | | + | + | | | + |
| 6.05 | | | | | | | | | | .. |
| 6.25 | + | ++ | + | .. | .. | + | + | | | .. |
| 6.35 | ++ | + | | | | | | | | |
| 6.50 | | + | .. | | | + | + | | | + |
| 6.70 | | + | | | | .. | .. | | | |
| 6.85 | | + | .. | | | | | | | |
| 6.95 | | + | | | | | | | | |
| 7.15 | | + | | | | | | | | |
| 7.25 | | + | | | | | | | | |
| 7.40 | | + | | | | | | | | |

++: intense bands, +: evident bands, ..: bands with weak intensity.
N: needle fascicle tissue, P: pollen
I-VIII: eight developmental stages of the male cone.

Twenty two isoforms of malate dehydrogenase with pI ranging from pH 3.75 to pH 7.40 were recorded during male cone development. Stage I of the male cone tissue showed the strongest enzyme activity and the most number of the isoforms, isoforms with pI 5.70; 6.95; 7.15; 7.25 and 7.40 were unique to the male cone tissue of this stage. Isoforms with pI 5.40 and 5.60 were unique to the pollen extracts. An isoform with pI 3.75 showed a strong intensity at stage II, III, V, VI and in pollen extracts. Isoforms with pI 4.25 and 4.55 showed a strong appearance at most stages of the male cone, including vegetative tissue and pollen extracts but were not present at stage VII and VIII. An isoform with pI 5 only showed its appearance from stage I to stage VI, disappeared at stages VII and VIII but reappeared in pollen extracts. An isoform with pI 5.05 was evident in vegetative tissue, stage I, II, III, V, VI and pollen extracts, but not in stage IV, VII and VIII. An isoform with pI 5.15 showed its appearance at vegetative tissue, stage I, disappeared at stage II, III and IV, reappeared at stage V and stage VI, disappeared again at stage VII and VIII, but reappeared again in pollen extracts. An isoform with pI 5.30 seemed only present in stages I, II, V, VI and pollen extracts but was not evident in vegetative tissue, stages III, IV, VII and VIII. The isoform with pI 5.80 showed its strongest appearance at stage I, decreased in stages II and III, disappeared at stage IV, came back strongly at stages V and VI, disappeared again at stages VII and VIII, but was present in pollen extracts. An isoform with pI 6.25 showed an intensely stained band in vegetative tissue, stages I and II, decreased at stages III and IV, came back strongly at stages V and VI, completely disappeared at stages VII and VIII, but reappeared again in pollen extracts. An isoform with pI 6.35 only existed in the vegetative tissue and stage I of the male cone tissue. Another isoform with pI 6.5 was evident in stage I, decreased in stage II, disappeared in stages III and IV, reappeared in stages V and VI and pollen extracts. It was not seen in stages VII or VIII.

Generally speaking, malate dehydrogenase was more active during the differentiation of the male cone than at any other stages of the male cone development. Because of their exclusive appearance in the selected stages of the male cone tissue and pollen extracts, but not in the vegetative tissue, isoforms with pI 3.75, 5, 5.30, 5.80 and 6.50 were more likely involved in the reproductive growth of the male cone. Another significant finding was that there was absolutely no sign of this enzyme activity at stages VII and VIII of the male cone tissue.

3. 3.4.4. Study of the Enzyme Activity of Peroxidase by Isoelectric Focusing

The enzyme activity of peroxidase was also studied with the method described in section 3.2.6, and the result is shown in figure 3.7. Analysis of the result is presented in Table **3.6.**

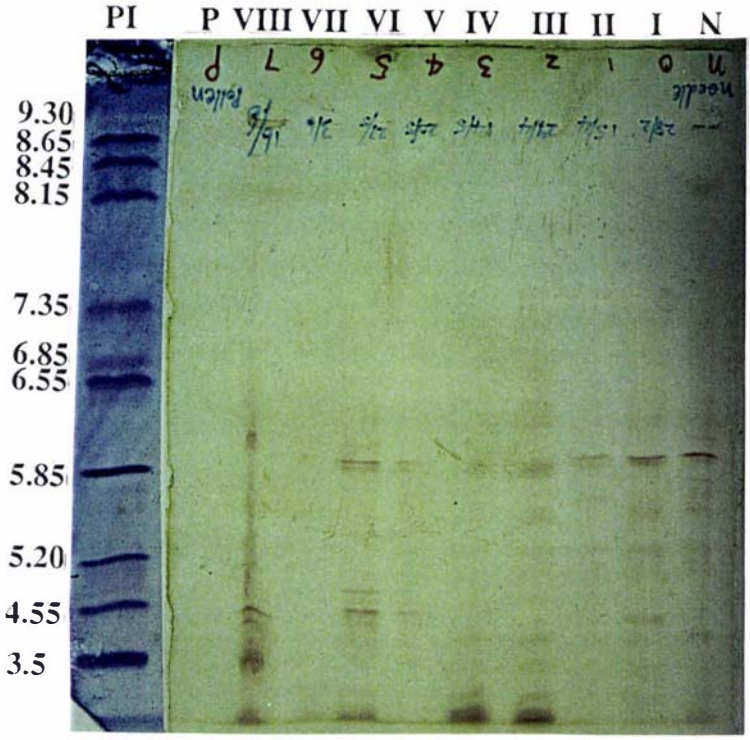



Fig 3.7. Isoelectric focusing gel electrophoresis study of the enzyme activity of peroxidase during male cone development in *Pinus radiata*. pI values were determined as described in the text (section 3. 2.6.4.). The numbers along the top of the figure refer to developmental stages described in Table **3.1**. N is needle tissue, P is an extract of mature pollen. The enzyme activity is expressed as red bands. See text for further detailed description of the gel.

Table **3.6.** Isoelectric focusing study of changes of the enzyme activity of peroxidase during male cone development.

| enzyme isoforms (pI) | N | I | II | III | IV | V | VI | VII | VIII | P |
|---|---|---|---|---|---|---|---|---|---|---|
| 2.15 | | | | .. | .. | | | | | |
| 2.95 | | | | .. | .. | | | | | |
| 3.20 | | | | .. | .. | | | | + | |
| 3.85 | .. | | | .. | .. | | | | + | |
| 4.35 | | .. | | | | .. | + | | + | |
| 4.70 | | | | | | | + | | | |
| 5.30 | .. | .. | | | | | | | | |
| 5.65 | .. | .. | | .. | | | | | | |
| 5.75 | | | .. | | | | | | | |
| 5.90 | + | | | | | | | | | |
| 6.00 | .. | .. | .. | .. | | .. | .. | | | |
| 6.10 | + | + | + | .. | .. | .. | + | | | |

++: intense bands, +: evident bands, ..: bands with weak intensity.
N: needle fascicle tissue, P: pollen
I-VIII: eight developmental stages of the male cone.

Twelve isoforms of the enzyme were revealed in the zymograph. Isoforms with pI 2.15 and 2.95 showed weak but clear appearance at stages III and IV of the male cone tissue. An isoform with pI 3.20 started to appear at stage III, decreased in intensity at stage IV, disappeared at stages V; VI and VII, reappeared strongly at stage VIII. An isoform with pI 3.85 was evident in the vegetative tissue and also appeared in stage III of the male cone. Its enzyme activity decreased in stage IV, was not seen in stages V; VI or VII, but reappeared with an intense band at stage VIII. An isoform with pI 4.15 showed clear banding at stages I; V; VI and VIII. An isoform with pI 4.70 only showed its existence at stage VI of the male cone tissue. An isoform with pI 5.90 was unique to the vegetative tissue. A final isoform with pI 6.10 appeared strongly in the vegetative tissue, stage I and II, disappeared at stages III and IV, but started to come back at stage V and showed its clear activity at stage VI.

The activity of peroxidase was not as intense as the other three enzymes examined in this study. There were no signs of enzyme activities in stage VII of the male cone tissue and pollen extracts.

## 3.4. DISCUSSION

After axillary buds of male cone-bearing branches differentiated into male cone buds, they were covered by spirally arranged microsporophyll primordia, as revealed by the anatomical study. During the development of the male cone, the male cone bud apex was gradually diminished by the acropetally developed microsporophylls, until male cone buds reached their maximum length, *i.e.* until the maximum number of microsporophylls had been formed. Once the microsporophylls were formed, microsporangial development started to occur. An increased number of sporogenous cells gradually filled up the microsporangium, and the enlarged microsporangium gradually occupied the greater part of the microsporophyll, apart from one or two cutinized layers of the epidermal cells. The enlarged microsporangium appeared to force the microsporophyll to grow outwards, until male cone buds reached their maximum width, *i.e.* until microsporangia reach their maximum size. These events have been illustrated in the previous light microscopy study.

From the description above it can be seen that male cone development is a development of microspophylls, a development of microsporangial tissue, and a development of pollen mother cells. Any morphological, anatomical, biochemical and genetical changes happening during male cone development are direct reflections of the male reproductive growth process, as the vegetative tissue becomes proportionally less significant in terms of contributing cytoplasmic material to whole cone extracts.

As the diameter of the male cone bud is more closely related to the size of the microsporangium, the steady increase of the average maximum diameter of male cone buds from stage I to stage IX, as shown in Fig 3.1, closely reflects the consistent growth of the reproductive tissue. When this reproductive growth advanced from stage I to stage II, however the general extractable protein content showed a sharp drop and decreased to its lowest level at stage VII, contrary to the steady increase in their size during this period of its development. Stage I corresponded to emergence of microsporophyll primordia. These tissues were largely made up of actively dividing meristematic cells, and it would be expected that more energy and resources are required to meet the needs of the rapidly increasing number of cells. It would also be expected that more protein enzyme production would occur to facilitate these processes. While stage II is when microsporophylls had already been well formed, there were not as many actively dividing meristematic cells as in the previous stage. The major activity at this stage is the

differentiation of sporogenous tissue in the basal microsporophylls of male cone buds. Stage VII was when pollen mother cells of pollen cone buds were at the pachytene stage of meiosis. Pollen mother cells which occupied most part of the microsporophyll were undergoing some significant biochemical and ultrastructural changes as described in Chapter Two. One of the most important of these was the elimination of ribosomes, described in the electron microscopy study at this stage. A similar event was also reported by Dickinson and Heslop-Harrison (1977) with their research on angiosperm species. They pointed out that the decrease in protein content could be a direct result of the reorganization of the cellular organelles, the elimination of ribosomes of pollen mother cells being a marker of preparation for the upcoming meiosis process.

The study of protein patterns by SDS-PAGE showed some significant changes in protein banding patterns, which revealed evidence for changes in gene expression during male cone development. A protein species with molecular mass of 20.5 KD (arrow 2, Fig 3.3) only existing in the male cone bud tissue at all stages, but not in the vegetative needle fascicle tissue, seems to be a male cone tissue specific protein, other protein species of 22.5 KD, 27.8 KD and 28.5 KD seem to be mature male cone tissue specific proteins, as they only showed their appearance in late mature male cone tissue where meiosis had occurred. These three protein species are probably only involved in the differentiation of the sporogenous tissue and meiosis process. Isolating and characterising these proteins could lead us to find those genes which are responsible for the appearance of these proteins, so that the genetic mechanism of this process could be better understood. Whether any of these protein species are expressed in developing female cones is unknown. One protein species with molecular mass of 17.4 KD showed a gradual change in its quantity: in vegetative tissue and stage I male cone tissue, there was only a trace amount of protein represented by a weakly stained band, but the protein content increased significantly from stage II male cone tissue, represented by a much more intensely stained band. The intensity of this band gradually decreased at stage VII and VIII of the male cone tissue, but was present in pollen extracts. These changes suggested that the nature of this protein species was probably enzymatic. The amount of this enzyme might be expected to increase and decrease as required by the differentiation and reorganization of the cellular and subcellular structures at particular stages of the male cone during its development, as it showed its strongest appearance at stage II male cone tissue, indicating that its major

role could be involved in the differentiation of the microsprangia. It is important however to emphasise that it is not possible to state with confidence that bands of a particular molecular weight in different gel lanes are exactly the same protein species.

It has been well documented that it is the continuous synthesis and/or degradation of specific enzymes and structural proteins that cause the cellular differentiations which bring about morphological development and functional specialization. SDS-PAGE can only provide general information on quantitative and qualitative changes in the denatured total protein, it is not able to provide any answers to changes of the native functional specific enzymes. With this in mind, native IEF gel techniques were applied in this study to analyse changes of four isoenzymes during male cone development in *Pinus radiata*.

Acid phosphatases ( AC, E.C.3.1.3.2) have been studied extensively during plant development. They are considered to play a role in the mobilization of phosphorus reserves during seed germination (Yamagata, *et al*, 1980), (Tamura, *et al*, 1982). More specifically, they are involved in the digestion of the endosperm during seed germination (Chandra Sekhar, *et al,* 1988).

In a gymnosperm species, Vanden Born (1963) found high concentrations of acid phosphatase localised at the bases of new needle primordia and in the provascular region in the stem in white spruce (*Picea glauca*). Fosket and Mikshe (1965) reported a high acid phosphatase activity in the potential apical initial and central mother cell zones in *Pinus lambertiana*. The first four lanes on the IEF gel of this study revealed the enzyme activity of acid phosphatase from pine tissue of four stages: young needle fascicle tissue, newly initiated male cone buds, male cone bud tissues where the microsporogenous tissue had just been differentiated, and male cone bud tissues where the microsporangia had just been formed. Five isoforms of this enzyme consistently showed a very strong activity at these four stages with pI ranging from pI 4.4-5.5. One extra isoform with pI 5 was found to begin its appearance at stage II and stage III and was possibly specifically activated with the differentiation of the early sporogenous tissue of the male cone. The intensely stained bands of these five isoforms and the appearance of the extra isoform from stage II clearly illustrated the strong involvement of enzyme activity of acid phosphatase at these early stages of the male cone development. This finding is in agreement with the conclusion drawn by Vanden Born (1963) and Fosket and Mikshe (1965) from their research in some

gymnosperm species, that the activity of acid phosphatase was primarily associated with tissues active in division or differentiation process during plant development.

Pennell and Bell (1986) found that soon after the intact tapetal cell layer was formed in the microsporangia of *Taxus,* acid phosphatase appeared in the plasma membranes of the tapetal cells. They suggested that these enzymes may lead to the generalized perturbation of the plasma membranes by dephosphorylating membrane phospholipid and phosphate-containing proteins. This enzyme then was probably responsible for the degradation of the tapetal cells at later stages. The detailed study by Dickinson and Heslop-Harrison (1977) on meiosis in angiosperms reported that the change in acid phosphatase activity in microspore mother cells in *Cosmos bipinnatus* had a correspondence with observed cytochemically-detectable ribonuclease, indicating that the enhancement of the ribonuclease activity from the acid phosphatase precedes the ribosome elimination phase at the pachytene stage of meiosis. On the IEF gel of this study, enzyme extracted from male cone bud tissue at stage VII, showed the maximum seven isoforms of acid phosphatase with a strong enzyme activity inferred from staining intensity of individual bands. Isoforms with pI 5.6 and pI 5.3 were especially heavily stained and were exclusive to this stage. This is when pollen mother cells of the male cone were in the late pachytene stage of meiosis, the number of cellular organelles had decreased significantly and the tapetal cells started to degrade. An increased enzyme activity at this stage would suggest the possible involvement of these acid phosphatase isoforms in the reorganization of ribosomes and other cellular organelles, and the degradation of the tapetal cells.

The activity of the acid phosphatase in the pollen grain wall has also been of a great interest to some researchers. Pettitt (1977) found that the intense acid phosphatase activity was associated with the intine at the sulcus in a primitive gymnosperm species, *Macrozamia reidlei.* He suggested that the localization of this enzyme in the aperture intine was an indication that this enzyme was activated in the metabolism of substrate encountered by the pollen tube during its growth through the nucellus. The same observation was also made in *Pinus* by Willemse and Linskens (1969), and in *Cycas* by Pettitt (1982). Heslop-Harrison *et al's* study (1973) on the pollen-wall proteins in the pollen walls of *Malvaceae,* together with evidence from other families, suggested that the intine-held proteins of angiosperm pollen grains were always produced by the male gametophyte, while those held in exine cavities were sporophytic in origin, being derived

from the tapetum. Acid phosphatase has been found only in the intine of pollen wall in *Malvaceae*, in *Brassica* (Vithanage and Knox, 1976), and some gymnosperm species (Pettitt, 1982). Thus, this enzyme has been shown to be a characteristic marker enzyme for proteins of gametophytic origin (Knox, *et al,* 1975).

Vithanage and Knox's work (1979) in sunflower, *Helianthus annuus L.* however showed that acid phosphatase was present in both intine and exine wall sites in contrast to their specific location in other pollen types. Quantitative cytochemical estimates of enzyme activity during the microsporogenesis of this species revealed that the enzyme patterns were characteristic of sporophytic origin, suggesting that the acid phosphatase was transferred from the tapetal layer onto both intine and exine wall sites of the pollen grain during the vacuolate period of the pollen. Clearly, there is still controversy on this point.

The IEF gel analysis of this enzyme from this study revealed a very similar banding pattern between vegetative tissue and pollen, indicating the likelihood of the sporophytic origin of most of the isoforms of acid phosphatase in pollen, but the banding patterns were also not exactly the same. There was an extra band in the pollen lane at pH 4.85, which was not present at any other stages of the pine tissue, suggesting expression of a gametophytic origin of this enzyme. In this study then, it is concluded that the acid phosphatase in radiata pine pollen may be either sporophytic or gametophytic in origin.

Non-specific esterase, (EST, E.C. 3.1.1.-): Vanden Born's work (1963) in white spruce (*Picea glauca*) showed that esterase activity could be detected in most parts of the shoot tip and terminal bud, but it showed its highest enzyme activity at the site where meristematic activity occurred most strongly, like young needle primordia and the young relatively undifferentiated sporophyll primordia. This result suggested that reactions catalyzed by esterase may play a prominent role in early cell differentiation as well as in cell division. The result of this IEF gel study is in agreement with this interpretation, the first three lanes, lane N, lane I, lane II showed not only a strong enzyme intensity but also showed the most number of the isoforms of this enzyme with different pI. These three lanes from the IEF gel of this study revealed the activity of this enzyme extracted from young needle fascicle tissue, newly differentiated male cone buds, and male cones with newly differentiated sporogenous tissue. Isoforms with pI 5.55 and 5.75 appeared to be

involved in both the initiation of male cone buds and the differentiation of the sporogenous tissue of the male cone, because of their exclusive appearance at stage I and stage II. Isoforms with pI 5.2, 5.3, 6.85, and 7 were probably involved in the differentiation of the sporogenous tissue of the male cone, because of their appearance at stages when the differentiation occurred. Isoforms with pI 6.85 and pI 7 disappeared at stage III and stage IV, but reoccurred at stage V and pollen extract. Stage V was when pollen mother cells started their prophase stage of meiosis. The appearance of these two isoforms at this stage and in pollen extracts, suggested that they were probably also involved in the meiosis process of pollen mother cells and the formation of the mature pollen.

It was not surprising to find a strong enzyme activity from the pollen extracts. There were 13 bands detectable on lane P (pollen) on the IEF gel, two of them showed an intensive enzyme activity at pH around 3.5; a number of bands only existed in this lane, but not in the previous stages. Esterase activity associated with pollen wall development has been reported for a number of plant species. In sunflower particularly, on releasing of the spores from the tetrad, esterase activity was found associated with the exine and the developing apertures. During the vacuolate period of pollen development, the enzyme activity was found in the intine and specially at aperture sites. During maturation the enzyme activity in the intine increased and the wall cavities showed esterase activity through to pollen maturity. The developing aperture of *Helianthus* pollen also had esterase activity associated with the thickening intine (Vithanage and Knox, 1979). In barley pollen, esterase was localised on the under- side of the aperture region, apparently external to the intine, suggesting that this esterase might be a cutin-hydrolysing enzyme, and might be implicated in self-incompatibility reactions on the stigma surface in barley (Christ, 1959). Vithanage and Knox (1979) pointed out that the presence of esterase in *Helianthus* pollen intine, especially at aperture sides, might be an indication of a gametophytic origin of different isoforms of esterase.

The substantial difference between lane N (needle) and lane P (pollen) of the banding pattern, and the lack of reaction on lane VI and lane VII on the IEF gel of this study showed good support for this interpretation, that is, the majority of the isoforms of esterase on the pollen wall are gametophytic in origin from the haploid microspore rather than sporophytic in origin from the diploid tapetum secretion.

Malate dehydrogenase (MDH, E.C.1.1.1.37) catalyses the reaction:

malate + $NAD^+$ = oxaloacetate + $NADH^+ + H^+$

Malate, as a very common organic acid found in every plant tissue, is mostly known as an important intermediary compound of the tricarboxylic acid (TCA) cycle. It is also a source of anions and protons. It contributes markedly to the water splitting process, and can be oxidized by plant mitochondria without control by the cell energy charge (Lance and Rustin, 1984). For these reasons, malate becomes involved in a wide variety of physiological processes, carbohydrate and lipid breakdown (Lance and Rustin, 1984), CAM and $C_4$ plant photosynthesis (Osmond and Holtum, 1981), maintenance of pH and electrical balance of the cytosol (Davies, 1979), resistance to anoxia (O'Leary, 1982), and stomatal movements (Willmer, 1983).

Through catalyzing malate as a substrate, MDH plays many important physiological roles within the plant cell. MDH is principally found in mitochondria, located in the mitochondrial matrix space (Nalk and Nicholas, 1985), and it can also be found in microbodies (glyoxysomes and peroxisomes), especially in fatty-acid plant tissues (fat-storing seeds) (Lance and Rustin, 1984).

In their detailed review of the role of malate in plant metabolism, Lance and Rustin (1984) drew a conclusion, in which they stated that malate was oxidized by MDH solely in plant mitochondria, and this enzyme mainly functioned in mitochondria. Mitochondrial MDH isoenzymes are also the best characterized of the organelle-associated isoenzymes (Scandalios, 1974). It has been reported that the recovery of nuclear gene mutations affected both catalytic efficiency and membrane binding ability of mitochondrial isozymes in *Neurospora* (Munkres *et al*, 1970). This result demonstrated that the structural genes for the mitochondrial isozymes were located in the nuclear rather than the mitochondrial genome. This conclusion was further supported by reports of different isozymes associated with different "mitochondrial populations" in heterotic barley hybrids (Grimwood, *et al* 1970). The use of antibiotic inhibitors of protein synthesis in *Neurospora* (Benveniste, 1970) suggested that the enzymes were synthesized on cytoplasmic ribosomes and subsequently incorporated into the mitochondrion, controlled by the nuclear structural genes (Scandalios, 1974). Based on these findings, a conclusion can be drawn that the fate of MDH is closely associated with the fate of mitochondria and ribosomes, if a significant drop or damage happens to the mitochondria and ribosome

population during certain developmental stages of the plant cell, the occurrence and the activity of MDH will be heavily affected.

Because of its important physiological functions within the plant cell, MDH isoenzymes exist in most plant tissue during the course of development. The native IEF gel analysis of the MDH isozymes during the male cone development from this study exhibited their appearance at most stages of the pine tissue. Not surprisingly, MDH extracted from stage I showed the maximum number of isoforms with heavily stained bands, a total of 17 isoforms were revealed at this stage with pI ranging from pH 4.25 to pH 7.40. This stage was when the newly differentiated male cone buds formed. The majority of plant cells at this stage are expected to be heavily engaged in division and differentiation processes. More energy and resources would be expected to be required from the TCA circle, potentially involving more isoforms and stronger enzyme activity of MDH at this stage of the male cone development. Five isoforms with pI 3.75, 5, 5.30, 5.80 and 6.50 only seemed to appear in certain stages of the male cone tissue and pollen extracts, but were not seen in the vegetative needle fascicle tissue, indicating that they were probably specific to male reproductive growth processes. Isoforms with pI 6.95, 7.15, 7.25 and 7.40 were unique to the male cone tissue of stage I, suggesting that they probably were only involved in the early differentiation process of the male cone tissue. Two isoforms with pI 5.4 and 5.60 were pollen specific. A striking finding from this study is that there was absolutely no enzyme activity of MDH detected in stage VII and stage VIII of the male cone tissue. These two stages were when pollen mother cells of male cone buds were undergoing a major reorganization of the cellular structure and organelles to prepare for the upcoming meiosis process. As revealed from the electron microscopy study from this project, the population of mitochondria and ribosomes in pollen mother cells and tapetal cells had decreased significantly, due to the proposed strong activity of the autophagic vacuoles at these two critical stages.

As the fate of MDH is so closely associated with the fate of mitochondrion and ribosomes, the disappearance of this enzyme activity at these two particular stages certainly provided a strong correlation to the finding made by transmission electron microscopy study of significant reduction in the mitochondria and ribosome populations at these two particular stages.

Peroxidase (PEX, E.C. 1.11.1.7) enzyme can utilize hydrogen to oxidize a wide range of hydrogen donors, such as phenolic substances, cytochrome C, nitrite, leuco-dyes, ascorbic acid, indole, amines, and certain inorganic ions, especially the iodide ion.

Peroxidases are widely distributed among higher plants, the richest known sources being the sap of the fig tree and the root of horseradish (Saunders, *et al,* 1964). Isoenzymes of peroxidase are known to occur in a variety of tissues in a large number of plant species. There is now evidence which indicates that peroxidases in plant cells are mainly located in cell walls, cytoplasm and vacuoles, depending in the last instance on the nature of the cell, and of its developmental stage (Griffing and Fowke, 1985), (Catesson, 1980). More specifically, Boller and Kende (1979) have reported that large amounts of peroxidase are located in cell walls (97%), while only an insignificant amount of peroxidase is located in vacuoles (1%) from tobacco suspension-cultured cells. In separate studies conducted over nearly four decades, peroxidase activity has been correlated both with the initiation of meristematic activity (Goff, 1975, Van Fleet, 1947), as well as with the suppression of growth (Lee, 1972; Ridge and Osborne, 1970), and terminal differentiation processes (e.g. tracheary elements) (Masuda, *et al.* 1983). A partial explanation for the apparently conflicting roles of peroxidase in organogenesis was considered to be expression of this enzyme in multimolecular forms, i.e. isoenzymes. In its participation in the homeostasis of IAA through its catabolism (Ros Barceló and Muñoz , 1992), it was suggested that peroxidase could play a crucial role in the reproductive development in plants, since IAA can both inhibit and promote flower initiation in flowering plants (Bernier, 1988). Kay and Basil's research in tobacco (1986) provided us with a detailed description of the correlation between the specific peroxidase isoenzyme activity and specific developmental events. Out of the 25 isoforms of peroxidase studied, they found that 3 were correlated with sustained cell division, 3 to 6 with lignification/tracheary element maturation, 7 with callus formation, 1 with localized suppression of growth, 3 with determinate axial organization, 4 with leaf development, and 1 with stamen development.

More closely related to the present study, Koul and Bhargava (1986) reported research results on the association of isoperoxidase with microspore differentiation in three plant species, *Carica papaya*, *Dioscorea composita* and *Ricinus communis.* Their results indicated that during the process of pollen mother cell meiosis in these three species, the variety of peroxidase isozymes was greater than during the pre- and post-meiotic stages of

microsporogenesis. While meiosis was in progress, five new peroxidases in *R.communis*, three in *D. composita*, one in *C. papaya* appeared, all these isoperoxidases disappeared as soon as meiosis was completed. Based on these results, they pointed out that the appearance of new isozymes indicated their specific role in pollen mother cells during meiosis and differentiation of microspores.

The native IEF gel study on the activity of isoperoxidase from the present study also detected some appearance and disappearance of certain isoforms of peroxidase during male cone development. An isoform with pI 6.10 showed a high activity band occurring in both vegetative fascicle tissue, and most stages of the male cone tissue, suggesting a nonspecific function in plant development, perhaps cell wall development. The disappearance of this band at stage VII and stage VIII, could be due to the state of pollen mother cells and tapetal cells of the male cone, as this is when pollen mother cells were engaged in the prophase stage of meiosis. Both pollen mother cells and tapetal cells were coated with a thick callose wall, the cellulose cell wall of pollen mother cells and tapetal cells were digested in most cases (Rowley and Walles, 1985). Thus the enzyme activity of peroxidase in relation to cell wall development was either not required or severely inhibited. Some specific isoforms were seen to occur only at particular stages of male cone development and were not seen in tissues at other stages. Isoforms with pI 4.70, pI 4.35, pI 3.85, and pI 3.20 only occurred in stage V and stage VIII. Stage V was when pollen mother cells of pollen cone were in the early stage of meiosis, stage VIII was when pollen mother cells of pollen cone were in the late pachytene stage of meiosis. The exclusive appearance at these two particular stages of the male cone of these four isoforms, suggested their specific functions were involved in the meiosis process of pollen mother cells of the male cone, possibly being involved in the reorganization of subcellular organelles and the cell wall differentiation in pollen mother cells.

In this work, stages of the male cone development in *Pinus radiata* were first characterized by cytological changes and then correlated to changes of the total soluble protein content, total soluble protein banding patterns and isoenzyme banding patterns. With the increase of the size of male cone buds, total protein content per unit dry weight showed a sharp drop before the mature pollen grain was released from male cone buds. SDS-PAGE study has shown differences in protein patterns, between the vegetative needle

tissue and the successive stages of male cone development. A number of protein species have been detected as potential male cone tissue specific gene expression products.

Native IEF gel study on four isoenzyme systems also showed some significant changes in their banding patterns between the vegetative needle fascicle tissue and the successive stages of male cone development. A number of isoforms of acid phosphatase, non-specific esterase, malate dehydrogenase and peroxidase have been shown to be potentially male cone tissue specific. Some of these are even stage specific only occurring at specific stages of male cone development. From these types of studies of the protein and enzyme patterns, it may be readily deduced that the occurrence of isozymes is a general phenomenon in all organisms and that they may provide a natural marker system for investigating a variety of problems in the genetics, biochemistry, and developmental biology of plants.

A summary of results from the SDS-PAGE and isoelectric focusing study, the specific occurrence of protein species with certain molecular mass, and isoforms of four isoenzymes expressed specifically in stages of male cone development is shown in Table **3.7.**

Markers of early reproductive development (I-IV: from the initiation of microsporophyll primordia to the formation of well developed microsporangia) are shown in blue bands. Markers of late reproductive development (V-VIII: meiosis process) are shown in red bands. Pollen markers are shown in dark yellow bands. Some bands are seen in both early and late reproductive growth. Some unique bands mark very specific stages of development only , and these are shown in brackets.

Table 3.7. The specific occurrences of some protein species and isoforms of four isoenzymes at different stages of male cone development.

| | N | I | II | III | IV | V | VI | VII | VIII | P |
|---|---|---|---|---|---|---|---|---|---|---|
| **Protein species KD** | | | | | | | | | | |
| **17.40** | | | — | — | — | | | | | — |
| **20.50** | | — | — | — | — | | | | | — |
| **22.50** | | | — | — | — | | | | | |
| **27.80** | | | | — | — | | | | | — |
| **28.50** | | | | — | — | | | | | — |
| **31.50** | | | — | — | — | | | | | — |
| **33.40** | | | — | — | — | | | | | — |
| **Isoforms of acid phosphatase pI** | | | | | | | | | | |
| **3.95** | | | — | | | | | | | |
| **4.85** | | | | | | | | | | (—) |
| **5.00** | | | — | — | | | | | | — |
| **5.20** | | | | | | | | | | — |
| **5.30** | | | | | | | | | | ( ) |
| **5.60** | | | | | | | | | | ( ) |
| **Isoforms of esterase pI** | | | | | | | | | | |
| **5.20** | | | (—) | | | | | | | |
| **5.30** | | | (—) | | | | | | | |
| **5.55** | | — | — | | | | | | | |
| **5.75** | | — | — | | | | | | | |
| **6.05** | | | | | | | | | | (—) |
| **6.85** | | | — | | | | | | | — |
| **7.00** | | | — | | | | | | | — |
| **7.25** | | | | | | | | | | (—) |
| **Isoforms of malate dehydrogenase pI** | | | | | | | | | | |
| **3.75** | | | — | — | | | | | | — |
| **5.00** | | — | — | — | — | | | | | — |
| **5.30** | | — | — | | | | | | | — |
| **5.40** | | | | | | | | | | (—) |
| **5.60** | | | | | | | | | | (—) |
| **5.70** | | (—) | | | | | | | | |
| **5.80** | | — | — | | | | | | | — |
| **6.50** | | — | | | | | | | | — |
| **6.95** | | (—) | | | | | | | | |
| **7.15** | | (—) | | | | | | | | |
| **7.25** | | (—) | | | | | | | | |
| **7.40** | | (—) | | | | | | | | |
| **Isoforms of peroxidase pI** | | | | | | | | | | |
| **3.20** | | | | | | | | | ( ) | |
| **4.15** | | | | | | | | | ( ) | |
| **4.35** | | | | | | | | | | |
| **4.70** | | | | | | | ( ) | | | |

It is clearly shown in Table **3.7** that different stages of the reproductive development is closely associated with expressions of protein species with different molecular mass and enzymes of different isoforms. Protein/enzyme markers revealed from this study provided us with valuable information about the biochemical process of male cone development in *Pinus radiata.*

Scandalios (1974) however pointed out that enzyme heterogeneity shown by the popular and convenient methods of electrophoresis or chromatography, resolved isozyme polymorphism mainly on charge or size difference, and this was not a sufficiently powerful technical tool for answering most genetic, biochemical, or physiological questions. He stated that mutations which inserted or deleted neutral amino acids from specific polypeptide chains were not likely to be detected by differences in mobility in an electric field. Yet such mutations might lead to altered physiochemical properties of the molecules which exert distinct controls on cellular metabolism.

Thus studies such as this reported here for *Pinus radiata* leave many questions unanswered. Isoelectric focusing analysis of isoenzyme yields more information than simple SDS-PAGE analysis, yet we can only make a tentative attempt to correlate the protein/enzyme patterns seen with developmental stage activity.

A more powerful technique at the molecular level is required to allow us to detect those minor alterations at gene level, which are likely to be associated with changes in gene expression. As a first step in dissecting these processes at a molecular level, the identification of genes likely to play a role in sex expression is necessary. The reminder of this thesis describes an experimental approach along these lines in the identification of members of a homeotic gene family involved in flowering in other angiosperms and gymnosperm plants.

# CHAPTER 4.0 A MOLECULAR BIOLOGY STUDY ON MALE CONE DEVELOPMENT IN *Pinus radiata*—A SEARCH FOR *PINUS* HOMOLOGUES TO GENES THAT CONTROL FLORAL DEVELOPMENT IN ANGIOSPERMS

## 4.1. LITERATURE REVIEW

Most information about the genetic control of floral development in higher plants is obtained through studies of flower development in angiosperms. The formation of flowers as reproductive structures is the characteristic feature of angiosperm species. Organ systems of the flower are found derived from cells originally present within the vegetative shoot meristem. During the process of plant development, the shoot meristem continuously produces leaves and stem until intrinsic or environmental factors signal the meristem to begin flower development. The induction of flower development results in reorganisation of the vegetative shoot meristem into a flower-producing floral meristem (Esau, 1977). Floral meristematic cells differentiate during flower development and are committed to the floral meristematic cell line upon division (Steeves and Sussex, 1972). Drews and Goldberg (1989) reviewed floral development and stated that "the conversion of a vegetative shoot meristem to a flower-producing meristem switches the plant from a continuous program of vegetative organ formation to a discontinuous pattern of floral development." They pointed out that in general, the floral organ systems of angiosperm plant species develop in whorls or helical rows from meristematic cells in a progressive order with sepals, then petals, then stamens, and finally carpels or pistils. Conifer species follow a similar pattern. Instead of continuously producing leaves and stems, the shoot apical meristem produces a simpler discontinuous floral organ system influenced by certain intrinsic or environmental factors. They don't have specified floral parts, such as sepal, petal, stamen and carpel. Instead, reproductive structures once produced are born on strobili or cones, which are terminal determinate structures comprising an axis with spirally arranged sporophylls. The microsporangia (presumed as an equivalent organ to anthers in angiosperms) are exposed on the surface of microsporophylls (presumed as an equivalent organ to stamens in angiosperms) which are spirally arranged on male cone axes in helical rows. Pollen is produced inside microsporangia. The ovules and seeds are

exposed on the surface of megasporophylls (presumed here as equivalent to carpels in angiosperms) or analogous structures, which are spirally arranged on the female cone axis. Generally speaking, as floral organ systems develop, meristematic cells are lost, and by the time the mature flower is established the floral meristem no longer exists (Drews and Goldberg, 1989). Experimental manipulation by Steeves and Sussex (1972) of the floral meristem in angiosperm species showed that there was a progressive and irreversible commitment of the primordia to develop into specific organ systems. At early stages of flower development, the floral meristem was found to be able to give rise to all floral organ systems. As flower development proceeded, primordia were committed to differentiate into specific organ systems. They also reported that surgical removal of a specified primordium (e.g. sepals) did not prevent the differentiation of other organ system primordia (e.g. petals and stamens), suggesting that floral primordia did not produce diffusible factors that induced the differentiated state of a contiguous primordium. Therefore, they concluded that the floral meristem had characteristics that were both regulative and mosaic in nature (Steeves and Sussex., 1972). It was reported that a vegetative shoot meristem could be induced to enter a flowering pathway by environmental signals such as temperature and day length, or by intrinsic factors such as age of the plant. Many plants were found to require a specific day length before they could enter the reproductive phase (Bernier, 1988). Physiological studies by Bernier (1988) suggested that photoperiods were perceived by phytochromes in leaves, and that a flowering signal was transmitted via an unknown signal response pathway to the shoot vegetative meristem. But some plants are day-neutral plants which do not require specific light conditions prior to flower induction. In these plants, flowering was found to occur after the plant reached a specific age; not determined necessarily by time but after a pre-set number of leaf nodes formed on the stem. Before this age the plant could not be induced to flower (Singer and McDaniel, 1986), (Gebhardt and McDaniel, 1987).

As to the flower induction mechanism, Bernier (1988) reported his grafting experiments with both day-neutral and photoperiod-sensitive plants. His experiments and those of many others showed that flower induction resulted in the translocation of a diffusible signal, known as florigen, from the leaf to the shoot meristem, that caused non-induced plants to flower. The chemical nature of florigen is still not clear, even after 50 years of research (Bernier, 1988). Recent experiments in both *Arabidopsis thaliana* and tobacco

(*Nicotiana tabacum*) indicate that florigen is not a cytokinin plant hormone (Medford *et al.*, 1989).

With regard to the genetic control of the floral development in plant species, Kamalay and Goldberg (1980) reported that each floral organ system expressed a unique set of genes. Their hybridization experiments with mRNA populations extracted from tobacco floral organ systems (petal, anther, ovary) showed that each organ expressed approximately 25,000 diverse genes. Both the anther and ovary contained approximately 10,000 diverse mRNAs that were not detectable in heterologous organ system mRNA or nuclear RNA populations (Kamalay and Goldberg, 1984). By contrast, Kamalay and Goldberg (1980) found that the petal contained approximately 7000 diverse mRNAs that were absent from all floral and vegetative organ system mRNA populations except the leaf. Drews and Goldberg (1989) pointed out that the qualitative similarity in petal and leaf mRNA populations probably reflected the homologous nature of these organs. They suggested that the differentiated state of each floral organ system was correlated with the expression of a unique set of genes, and that transcriptional events probably played a role in regulating gene expression during flower development.

Gene expression during flower development was also reported to be regulated temporally and spatially. *In situ* hybridisation experiments by Gasser *et al* (1988) demonstrated that many organ-specific mRNAs were present within specific cell or tissue types. For example, Goldberg (1988) reported that some mRNAs were only detectable in the tapetum (a cell layer that synthesises molecules required for pollen development). They accumulated early in anther development when the tapetum was active, and decayed when the pollen was mature and the tapetum was destroyed.

These experiments indicate that cell specification events which occur during the differentiation of floral organ system primordia are the result of the activation of specific gene sets in a precise temporal framework. The molecular processes that control the expression of specific gene sets during flower development were unknown until the discovery of homeotic mutants in plant development.

Homeosis in plants and the fact that individual homeotic genes are responsible for homeotic phenotypes has been recognised for a long time. The older literature has been reviewed by Coen (1991). The initial understanding of homeotic genes and their operation was based on studies of *Drosophila melanogaster* homeotic mutants (French *et al.*, 1988;

Ingham, 1988). These studies identified genes that regulated the positional organisation of cells during embryogenesis, and found that many of these genes acted as transcription factors, suggesting that such homeotic mutations at individual genetic loci must necessarily have occurred in genes whose function was to activate or co-ordinate the myriad of other genes whose combined expression was required to contribute to the formation of a specific organ.

In plants, a number of homeotic mutations have been identified which altered the specification of floral organs. *Arabidopsis thaliana (L.) Heynh.* has been used as a model plant to study the molecular mechanisms of flower development in plants. *Arabidopsis*, without further qualifications, in this context, will refer to *Arabidopsis thaliana (L.) Heynh.* in its diploid form (2n = 10).

Koncz *et al* (1992) in reviewing recent progress in *Arabidopsis* research stated that the general molecular mechanisms that were responsible for the physiological, cellular and biochemical properties of plants would be expected to be essentially conserved in all plants, and these mechanisms should also operate in *Arabidopsis.* Hence, they pointed out, that its genome should contain most of the genes that control the genetic mechanisms of the life processes in general plant species. This comment indicates that studying the molecular mechanisms of *Arabidopsis* can help us to understand the genetic determination of developmental processes of any other plant species. The reason *Arabidopsis* is chosen as a model plant is because of its unique genome features. The *Arabidopsis* genome is unusual both for its small size and for its near-absence of interspersed repetitive DNA. Based on the current knowledge at the time, Meyerowitz (1992) summarised that the total *Arabidopsis* genome size is ~80,000-100,000 kb, [most flowering plants' haploid genome sizes are several- to five hundred-fold higher than that of *Arabidopsis* (Meyerowitz and Pruitt, 1985)] with highly repeated and moderately repeated DNA comprising together only about 20% of the genome. The low-copy component of the genome is ~65,000-80,000 kb, and he presumed that it contained virtually all of the protein-coding DNA, including single-copy genes and those genes that existed in families of two to ten or so copies.

Even though the selective advantage of such an unusual genome size is not clear, Meyerowitz stressed the important practical advantage of dealing with small amounts of

nuclear DNA and repetitive DNA in experiments in molecular biology, such as gene cloning and chromosome walking (Meyerowitz and Pruitt, 1985).

Because of the unique genome size and organisation in *Arabidopsis,* most of the homeotic genes controlling the development of floral meristem and floral organs were firstly identified and characterised in this species. Yanofsky (1995) and Jordan and Anthony (1993) reviewed the latest progress on studies of floral homeotic genes during floral development. They stated that during the transition from vegetative growth to flowering, two stages could be recognised. The first stage (early stage) was the alteration of the apical meristem either directly to a floral meristem or in some cases to an inflorescence meristem and subsequently to a floral meristem. The second stage (late stage) was the development of the reproductive organs; organogenesis. Jordan and Anthony (1993) concluded that both of these stages could be considered to be homeotic changes as they involved the replacement of meristem or organs by other types. Schwarz-Sommer *et al* (1990) classified the homeotic genes involved in both of these phases as either early genes or late genes due to their expression.

The majority of these homeotic genes contain a highly conserved "MADS-box" region. The proposed molecular functions of these homeotic genes were extensively characterised through studies of the mutant phenotype in *Arabidopsis.* Table **4.1** shows a number of homeotic genes that play an important role in the regulation of floral meristems and floral organs in *Arabidopsis*, and genes identified from other plant species which are homologous to those identified in *Arabidopsis.*

Table **4.1**. Genes Involved in the Regulation of Meristem and Floral Organ Identity in *Arabidopsis* and Their Homologous Genes Identified from Other Plant Species.

| Genes of *Arabidopsis* | Mutant Phenotype | Proposed Molecular Function(s) | Putative Homologous Genes from other species |
|---|---|---|---|
| **Floral Meristem Identity** | | | |
| *LEAFY (LFY) (1)* | Partial conversion of floral meristems to inflorescence shoots | Putative transcription factor, positive regulator of *AP3* and *PI* | *FLORICAULA (FLO)* (2) |
| *APETALA1 (AP1)* (3) | Production of flowers that have partial inflorescence character in that secondary floral meristems arise in the axils of the first whorl organs (sepals). | Putative transcription factor | *SQUAMOSA (SQUA)* (4) *OsMADs1* (5) |
| *APETALA2 (AP2)* (6) | Similar to *ap1* under short day growth conditions. | Negative regulator of *AG* | Not reported |
| *CAULIFLOWER (CAL)* (7) | *ap1 cal* double mutants display a conversion of the floral meristem to an inflorescence | Unknown | Not reported |
| **Mediators between Floral Meristem Identity and Organ Identity Genes** | | | |
| *AGL-2, AGL-4, AGL-6 (8) and AGL-9 (9)* | Unknown | Putative transcription factors, express after *LFY* and *AP1* but before *AP3*, *PI*, and *AG* | *FBP2 (10), DAL-1 (11)* |
| **Floral Organ Identity** | | | |
| *APETALA1 (AP1) (3)* | Homeotic conversion of sepals to leaves; absence of petals | Putative transcription factor | *SQUAMOSA (SQUA)* (4) *OsMADs1* (5) |
| *APETALA2 (AP2) (6)* | Homeotic conversion of sepals to leaves or carpels and of petals to stamens. | Negative regulator of *AG* | Not reported |
| *APETALA3 (AP3) (12)* | Homeotic conversion of petals to sepals and of stamens to carpels | Putative transcription factor and positive regulator of *PI* | *DEFICIENS (DEF) (13)* |
| *PISTILLATA (PI) (14)* | Similar to *ap3* | Putative transcription factor | *GLOBOSA (GLO) (15)* |
| *AGAMOUS (AG) (16)* | Homeotic conversion of stamens to petals and of carpels to sepals | Putative transcription factor, negative regulator of *AP1*, *AP2*, and *AP3* | *PLENA (PLE) (17) BAG 1(18), TAG1(19), ZAG1, ZAG2 (20), OM1(21), and DAL-2 (11)* |
| *AGL-1* and *AGL-5* (8) | Unknown | Target genes of *AG* | Not reported |

Genes containing MADS-box are underlined. (1) Weigel *et al.*, (1992); (2) Coen *et al.*, (1990); (3) Mandel *et al.*, (1992 a); (4) Huijser *et al.*, (1992); (5) Chung *et al.*, (1994); (6) Jofuku *et al.*, (1994); (7) Bowman (1992); (8) Ma *et al.*, (1991); (9) Mandel and Yanofsky, unpublished data; (10) Angenent *et al.*, (1994); (11) Tandre *et al.*, (1995); (12) Jack *et al.*, (1992); (13) Sommer *et al.*, (1990); (14) Hill and Lord (1989); (15) Tröbner *et al.*,(1992); (16) Yanofsky *et al.*, (1990); (17) Bradley *et al.*, (1993); (18) Mandel *et al.*, (1992 b); (19) Pnueli *et al.*, (1994); (20) Schmidt *et al.*, (1993); (21) Lu *et al.*, (1993).

From Table **4.1**, it can be seen that most floral homeotic genes contain a MADS-box region, so they are also called MADS-box genes. The MADS-box genes are underlined in Table **4.1**.

The first homeotic gene to be isolated that was expressed at an early stage of floral morphogenesis was *FLORICAULA* (*FLO*) by Coen *et al* (1990) although it did not contain a MADS-box region. *FLO* was isolated from a *floricaula* mutant of *Antirrhinum majus* created by transposon mutagenesis. *In situ* hybridization techniques and PCR-based transcript measurements confirmed that *FLO* was expressed as a 1.6 kbp transcript at an early stage of wild type floral development. From this study, Coen and his colleagues suggested that the *FLO* gene could play a role in the switch to the floral meristem from the vegetative meristem and in organogenesis by expressing proteins that switch on organ identity genes. The *Arabidopsis leafy*1(*lfy*1) mutant reported by Weigel *et al* (1992) had a similar phenotype to the *floricaula* mutant in *Antirrhinum majus.* They found that the *LEAFY* (*LFY*) gene was also expressed in a similar pattern to *FLO* with the strongest expression in the young flower primordia surrounding the inflorescence meristem. But differences in gene expression were found at later stages as reported by Huala and Sussex (1992), who found that *LFY* was expressed in stamens whereas *FLO* was not. Another difference was that strong *LFY* mutants were not completely transformed into inflorescence morphology unless *LFY* was combined with another homeotic gene isolated from *Arabidopsis APETALA1-1* (*AP1*) or *APETALA2-1*. The *AP*1 gene was cloned and was found to be a new member of the "MADS-box" family of genes (Mandel *et al.*, 1992a). *AP1* mRNA was detectable in young flower primordia as soon as they were visible on the flanks of the inflorescence meristem but not in the inflorescence meristem. The expression of *AP1* began to decrease in the cells of the two inner whorls (stamen and carpel whorls), and at all later stages of flower development. *AP1* mRNA was only detected in sepal and petal primordia. The molecular and genetic data from their study supported the conclusion that *AP1* acted locally to specify the identity of floral meristems and to define pedicel tissue as floral rather than inflorescence and thus to suppress meristem formation, but its function appears to be restricted to the early stages of flower development and the two outer whorls sepal and petal primordia (Mandel *et al.,* 1992b).

Another homeotic gene in *Antirrhinum majus* which is involved in the early stages of floral development, *SQUAMOSA* (*SQUA*) was reported by Huijser *et al* (1992). They

found that *SQUA* gene expression was detectable as soon as floral primordia were present and was expressed in the cells giving rise to the floral organs. Although *SQUA* expression appeared to be important in the early stages of development and in organ identity, Huijser *et al* (1992) pointed out that the expression of *SQUA* was not essential and its function could be carried out by some other gene product. Another important character of this gene was that it is a member of the MADS-box gene family (Huijser *et al*., 1992).

Most homeotic genes isolated so far are expressed at later stages of flowering and control the determination of each floral part. Sommer *et al.* (1990, 1991) reported a gene called *DEFICIENS* which was expressed at a later stage of floral development in *Antirrhinum majus*. Its expression was limited to organ primordia and was not found in earlier stages. In *Arabidopsis*, the *APETALA3* gene was shown to be homologous to *DEFICIENS* both at the level of the mutant phenotype and the DNA sequence (Jack *et al*., 1992). *In situ* hybridisation experiments revealed that mRNA transcripts of the *DEFICIENS* gene were abundant in petals and stamens as well as in nonsporogenic tissue such as the filament, connective tissue, epidermis and endothecium of the anthers. Low levels of *DEFICIENS* gene expression were also detected in the gynoecium. The level of the *DEFICIENS* mRNA transcripts was relatively stable once established in a particular organ. Schwarz-Sommer *et al* (1992) concluded that persistent expression of the *DEFICIENS* gene during organogenesis, combined with that of two other genes (*OVULATA* and *PLENA*), specified the identity of the petals and stamen of *Antirrhinum*, respectively .

In addition to the *Antirrhinum majus* studies, the role of homeotic genes in organogenesis (late stage) was extensively investigated during *Arabidopsis* floral development. As with *Antirrhinum majus*, the research focused on mutant phenotypes to characterise the roles of homeotic genes in the determination of organ identity. Jordan and Anthony (1993) reviewed these results and presented a clear picture of how the related homeotic genes control the identity of each floral organ during flower development in *Arabidopsis*. In their review, mutants of *Arabidopsis* were placed in three general groups. The first group, represented by *APETALA2*, altered the outer two whorls, thus sepals were transformed into carpels and petals into stamens. *APETALA3* and *PISTILLATA* represented the second group and affected the transformation in the second and third whorl, transforming petals into sepals and stamens into carpels. The third group, represented by *AGAMOUS* (*AG*), influenced the development of the third and fourth whorls and transformed stamens into

petals and carpels into sepals (Jordan and Anthony, 1993). Bowman and Meyerowitz (1991) pointed out that *AGAMOUS* RNA was restricted to specific cell types within the stamens and carpels as cellular differentiation occurred in those organs, and the early expression of the *AG* gene was regulated by *APETELA2* (*AP2*), but the late *AG* expression was not directly dependent on *AP2* activity.

Yanofsky (1995) recently reviewed the important roles of MADS-box genes in flower development in *Arabidopsis*. He introduced an "ABC" model to characterise homeotic organ identity genes which control three different activities, designated A, B, and C, to specify the four different organ types. In his model, each of these homeotic activities functioned in two adjacent whorls, with A in Whorls 1 (sepals) and 2 (petals), B in whorls 2 (petals) and 3 (stamens), and C in whorls 3 (stamens) and 4 (carpels ). He stated that activity A alone in whorl 1 specified sepals, and C alone in whorl 4 specified carpels. The combined activities of AB and BC specify petals and stamens, respectively, in whorls 2 and 3. His model further suggested that A and C were mutually antagonistic, suggesting that A prevented the activity of C in whorls 1 and 2, and C prevented the activity of A in whorls 3 and 4. This model clearly demonstrated how the determination of the identity and spatial location of the organ on the floral meristem could be controlled by a series of homeotic genes, working either alone or in combination to ensure that floral organs develop in a correct order.

Following the successful cloning and sequencing of *DEFICIENS*, it was found that the putative protein sequence revealed a 227 amino acid reading frame with a conserved domain showing homology to known transcription factors. The *AGAMOUS* protein product was similarly found to exhibit this sequence conservation, and consisted of 55 amino acid residues with extensive similarity to transcription factors found in humans, eg. serum response factor, *SRF* (Norman *et al.*, 1988) and yeast, *MCM1* (Jarvis *et al.*, 1989; Ammerer, 1990). In humans, the serum response factor (*SRF*) was thought to be required for the serum-inducible transcriptional activation of genes such as *c-fos*, a nuclear proto-oncogene of mammals (Norman, *et al.*, 1988). In yeast, the product of the *MCM1* gene was thought to be a transcriptional regulator of mating-type-specific genes (Jarvis *et al.*, 1989; Ammerer, 1990). On the basis of their sequence similarity and their phenotype, it was proposed that the *AGAMOUS* (Yanofsky *et al.*, 1990) and *DEFICIENS* (Sommer *et al.*, 1990) proteins are transcription factors involved in regulating genes that determine

stamen and carpel development in wild-type flowers. The conserved domain shared by these four proteins was called the "MADS-box" in reference to the four founding proteins (MCM1, AGAMOUS, DEFICIENS and SRF). The MADS-box represents a highly conserved motif within the N-terminus region (Yanofsky et al., 1990). The alignment of amino acid sequences deduced from conserved MADS-box sequences of *MCM1* gene from yeast (Passmore *et al* ., 1988), *AGAMOUS* gene from *Arabidopsis* (Yanofsky *et al.*, 1990), *DEFICIENS* gene from *Antirrhinum majus* (Sommer *et al.,* 1990) and *SRF* gene from humans (Norman *et al.*, 1988) is shown below: Highlighted amino acids show differences between AGAMOUS MADS-box domain and three other amino acid sequences.

| | |
|---|---|
| AGAMOUS | RGKIEIKIRENTTNRQVTFCFRRNGLLKKAYELSVLCDAEVALIVFSSRGRLYEY |
| DEFICIENS | RGKI**Q**IKIREN**Q**TNRQVT**YS**FRRNGL**F**KKA**H**ELSVLCDA**K**V**SI**I**MI**SS**TQK**L**H**E**Y |
| MCM1 | R**R**KIEIK**F**REN**KT**R**R**H**V**TF**S**FR**KH**G**IM**KKA**F**ELSVL**TGTGV**L**LL**V**VS**ET**G**LVYTF** |
| SRF | R**V**KI**KMEFI**DN**KI**R**RYT**TF**S**FR**KT**G**IM**KKAYELS**TLTGTGV**L**LL**V**AS**ET**G**HVYTF** |

The functional roles of the MADS box genes have been studied by several different approaches. When appropriate mutants and stable transformation systems are available, genetic complementation tests can be used to elucidate the role of a MADS box gene. Meyerowitz's laboratory showed that a genomic clone corresponding to the *Arabidopsis AGAMOUS* gene complemented the mutation, resulting in the generation of wild-type flowers (Yanofsky, *et al.*, 1990).

Using a conserved DNA binding sequence within the MADS-box as a hybridisation probe, Angenent *et al.* (1992) isolated and characterised two flower-specific genes from *Petunia hybrida* (*Petunia*). They found that the protein product of these genes, designed Floral Binding Protein1 (FBP1) and 2 (FBP2), had two regions of relatively high homology. The amino acid sequence was highly conserved between FBP1, FBP2 and other known MADS-box domains and a potential phosphorylation site common to all DNA-binding domains was present. The *FBP1* gene was shown to be expressed exclusively in the petals and stamens of *Petunia* flowers, although the protein was only detectable in the petals. The *FBP2* gene was expressed in petals, stamens, carpels and at a very low level in sepals (Angenent *et al* ., 1992). Kush *et al.* (1992) have also isolated homologues of the *DEFICIENS* gene from *Petunia*. Transgenic *Petunia* plants were generated which

constitutively expressed the *DEFICIENS* homologue gene product under the control of the CaMV 35S promoter. Kush and his colleagues' study showed that the expression of the homologue *DEFICIENS* gene product in sepals of *Petunia* led to a partial conversion of sepals into petals (Kush *et al.*, 1992).

Five genes with homology to *DEFICIENS* and *AGAMOUS* were isolated from tomato, (*Lycopersicon esculentum*) by Pnueli *et al* (1991). They found that these five genes represented regulatory genes of the MADS-box family and individual genes were expressed during meristematic and late floral programmes. cDNA libraries were constructed from mRNA of mature tomato flowers, and the libraries were screened with the *Antirrhinum DEFICIENS* cDNA clone in their study. Twelve clones representing five genes were isolated by virtue of their partial homology with the *DEFICIENS* gene of *Antirrhinum*. They found that four of the tomato genes were flower-specific with distinguishable temporal expression. *TM4* and *TM8* expressed in the early floral development, while *TM5* and *TM6* expressed in the late floral development. Each of the genes were unique in the genome and could be localised to different chromosomes by RFLP mapping. They reported that it was now apparent that more than a dozen MADS genes existed in tomato (Pnueli *et al.*, 1991). Pnueli *et al* (1994) also generated transgenic tomato plants expressing antisense RNA of the tomato *TAG1* (*AGAMOUS* gene homologue in tomato) and showed that the reduction of the mRNA level of this MADS box gene affected organ development in the two inner whorls, resulting in the conversion of stamens into petals and of carpels into indeterminate nested sets of perianth flower (Pnueli *et al.*, 1994). His study suggested that a reproductively sterile plant could be generated with this approach by disrupting the expression of a particular floral-specific homeotic gene.

Ectopic expression of MADS box genes in floral organs has also generated substantial information on the functional mechanism of these regulatory genes. Similar approaches were used to study the functional roles of *AGAMOUS* homologues isolated from a variety of other plants including *AGL1-AGL6* genes from *Arabidopsis thaliana* (Ma *et al.*, 1991), *BAG*1 gene from *Brassica napus* (Mandel *et al.*, 1992b), and *TOBMADS*1 gene from tobacco, *Nicotiana tabacum* (Mandel, T. *et al.*, 1994).

Ma *et al* (1991) studied the functional roles of the *Arabidopsis AG*-like genes, *AGL*1-6. They found that five of the *AGLs* were expressed preferentially in flowers and young pods

but not in leaves or stems. At this level, they were similar to *AG* from *A. thaliana* and *DEFICIENS* (*DEF A*) from *A. majus*. On the basis of sequence comparison, they put *AG*, *AGL-1*, and *AGL-5* in one subfamily and *AGL*-2, *AGL*-4 and *AGL*-6 in another family. They also found that the expression patterns of *AGL*-1 and *AGL*-2 within the flower were slightly different from that of *AG* . The onset of *AGL*-1 and *AGL*-2 expression was much later than that of *AG*. *AGL*-1 was expressed preferentially in carpels, not in stamens, petals, or sepals. The *AGL*-2 signal was found primarily in carpels and was lower in stamens. In carpels, they detected that the expression of both *AGL*-1 and *AGL*-2 was concentrated in ovules. They suggested that *AGL*-1 and *AGL*-2 were probably involved in regulating ovule development (Ma *et al*., 1991). This conclusion indicates that *AGL*-1 and *AGL*-2 are probably involved in female floral organ development.

Three years later, a more detailed characterisation of the expression pattern of *AGL*-2 was published by Flanagan and Ma (1994) which brought up a more complete description of the expression pattern of *AGL*-2. They reported that *AGL-2* was first expressed very early in development, before any of the organ primordia emerged, but after the floral meristem emerged from the inflorescence meristem. They also found that *AGL*-2 transcript was very abundant and uniform throughout the floral meristem and in the primordia of all four floral organs: sepals, petals, stamens and carpels. They pointed out that *AGL*-2 represented a new class of MADS-box genes which was expressed in all four whorls of the flower, and the *AGL-2* transcript remained abundant in each organ during morphological differentiation, but diminished as each organ underwent the final phase of development (Flanagan and Ma, 1994).

*AGL*-2 and *AGL*-4 were also recently reported to initiate after *LFY* and *AP*1 but before *Ap*3 and *AG,* Yanofsky (1995) suggested these two genes could function as mediator genes between early meristem and late organ identity genes. He also stated that as *AGL*-5 required *AG* for its expression and its RNA began to accumulate shortly after that of *AG*, *AGL*-5 probably was a candidate target gene of *AG* (Yanofsky 1995).

Isolating DNA sequences related to MADS-box genes by PCR has been tried with success in a monoecious species, birch (*Betula pendula* Roth.) by Tikka *et al*., (1993). By using degenerative oligonucleotides corresponding to two well-conserved sequences in the MADS-box region, they amplified by PCR approximately 95 bp long regions of birch

genomic DNA. The amplification products were cloned into M13 vector and clones containing the MADS sequence were identified.

The sequencing of 13 MADS-box containing M13 clones revealed 7 different clones. When they compared their nucleotide sequences with one another, these clones were divided into 2 groups, one group having an identity of 88 - 92% to the MADS-box of *Antirrhinum majus DEFICIENS* gene and the other group having a 91% identity to the MADS-box of *Arabidopsis AGAMOUS* gene. Based on these results, they stated that birch (*Betula pendula*) contained a large family of MADS-box genes.

Additionally, they screened a birch genomic DNA library, using an *Arabidopsis AGAMOUS* cDNA clone as a probe. One genomic clone was isolated and partially characterised. They found that the first exon of the gene encoded a MADS-box having 92% nucleotide sequence identity to the MADS-box of the *Arabidopsis AG* gene (Tikka, *et al.,* 1993). More recently, they have detected some strong signals in Northern hybridisations between an *Arabidopsis AGAMOUS* cDNA probe and RNA samples isolated from different developmental stages of birch (personal communication).

It appears that flower development may be controlled in similar ways in dicotyledon and monocotyledon plant species since MADS box genes were also isolated from several monocotyledon species including maize (*Zea mays*) (Schimidt, *et al.*, 1993), orchid (*Aranda deborah*) (Lu *et al.*, 1993), and rice (*Oryza sativa*) (Chung *et al.*, 1994). Schmidt *et al* (1993) isolated two maize MADS box genes, *ZAG1* and *ZAG2* (*AGAMOUS* homologues in *Zea mays*). They reported that throughout the protein coding region, the amino acid conservation between *ZAG1* and *AGAMOUS* was about 61% and the MADS boxes were identical except for two conservative amino acid substitutions. The *ZAG1* transcript accumulated early in stamen and carpel primordia, resembling the expression pattern of *AGAMOUS*. Mapping experiments revealed that *ZAG1* was located near the polytypic ear locus (pt) which was known to affect maize flower development (Schmidt *et al.*, 1993).

Most recently, using the PCR technique, several hundred thousands of the maize clones were screened with a set of specially designed primers (targeting the region between the insert transposon and part of the *ZAG1* sequence) and one clone with a disrupted *ZAG1* gene was identified. Self-crossing of this clone generated *ZAG1* mutant maize progeny

with phenotypes similar to the *Arabidopsis AGAMOUS* mutant (Schmidt *et al.*, personal communication). This experiment again confirmed that *ZAG1* gene is *AG-like* and floral specific, controlling the stamen and carpel development in *Zea mays*, since a maize clone with unfunctional floral organs could be generated by disrupting the *ZAG1* gene.

Gymnosperm species have reproductive organs that differ from angiosperm flowers in fundamental respects. The gross differences in organisation of seed cones versus angiosperm inflorescence, the lack of a carpel surrounding the ovules in gymnosperms, and the spiral, rather than whorled, arrangement of pollen-bearing organs are some examples listed by Strauss *et al* (1995). They also bear reproductive axes that only produce organ primordia for one sex, and the control of this type of unisexuality depends upon alteration of primordium identity, which is different from most angiosperm species. However, gymnosperm species are still seed plants, and they also have some features in common with angiosperms (e.g. the presence of a tapetum that nourishes pollen during development, see the anatomical study of this thesis).

Conifers, as the largest group in the gymnosperms, include most of the forest species on earth. Their reproductive growth pattern was reviewed by Owens (1985). As they reach reproductive age, seed cones (female cones) are produced first, usually on vigorous first order or second order leading shoots located in a zone at the top of the adult tree, followed by pollen cones (male cones) on less-vigorous third or fourth order branches located further down the adult tree crown. Reproductive buds of most northern conifer species undergo early development before winter dormancy and overwinter at various stages. Pollination occurs in the spring or early summer of the second year and seed maturation occurs in the same year as pollination or the year after. Mature seeds do not have the protection from the ovary structure like angiosperm species (Owens 1985). Even though conifer species have significant differences in the development and structure of the floral organ from those of angiosperm species, recent research suggests that the control of the reproductive development is similar to that in angiosperm species.

It is now clear that many angiosperm floral genes, including transcription factors, have homologs that can be readily identified and used for engineering sterility in gymnosperms once their modes of expression are known (Strauss *et al.*, 1995). Several successful

attempts have been made to examine the possibility of MADS-box gene control of reproductive identity in gymnosperm species.

Strauss and his colleagues isolated a *LEAFY*-like gene from Douglas-fir (Nyers *et al.*, 1993). Rotledge *et al* (1993) isolated putative homeotic genes from black spruce (*Picea mariana*) which were homologous to the *Arabidopsis thaliana AGAMOUS* gene and the *Zea mays Knotted1* gene. Rotledge *et al* (1993) used two degenerate primers targeting highly conserved regions within the MADS-box, and amplified a 60 bp segment from multiple MADS-box gene members. Combined with routine subcloning into M13 and sequence analysis of individual subclones, they have identified forty distinct DNA fragments containing a MADS-box region. From this finding, they suggested that black spruce had a large and complex MADS-box gene family which might contain over 100 MADS-box related genes.

Based on the comparison of these DNA sequences, they suggested that the black spruce MADS-box gene family can be subdivided into 10 to 15 subfamilies, and they found that one of this subfamily shared extensive identity of the deduced amino acid sequence to *AGAMOUS*; another shared high identity to *SQUAMOSA* [a MADS-box gene in *Antirrhinum* involved in early stages of floral development (Huijser *et al.*, 1992)], several others were highly distinctive and appeared to be unrelated to any other previously characterised MADS-box genes.

They also reported that a large proportion of the identified MADS-box genes were transcribed, and one *AGAMOUS*-like gene was predominantly expressed in somatic proembryos, when they did a PCR cloning from cDNA derived from black spruce embryogenic cultures (Rutledge, *et al.*, 1993).

The most thorough study of floral homeotic genes from conifer species was reported by Tandre *et al* (1995). They isolated three different *DEFICIENS-AGAMOUS*-like genes called, *DAL-1*, *DAL-2* and *DAL-3* in Norway spruce, (*Picea abies*). Using DNA sequence comparisons they found that *DAL1* was related to *AGL2*, *AGL4* and *AGL6* from *Arabidopsis thaliana* which are members of the MADS-box gene family, are preferentially expressed in flowers and which probably act to control many steps of *Arabidopsis* floral morphogenesis. Likewise, *DAL-2* was closely related to its angiosperm counterparts, the *AGAMOUS* and *DEFICIENS* genes, that control the identity of sexual organs (stamens and carpels in angiosperm). *DAL*-2 was expressed only in the developing male and female

cones in *Picea abies*. *DAL3* was related to the vegetatively expressed tomato MADS-box *TM3* gene and was transcribed in both vegetative and reproductive shoots. Tandre *et al* (1995) stated that " The functional and structural complexity within the MADS-box superfamily of reproduction-control genes is a conserved ancestral property of seed plants and not a novelty in the angiosperm lineage". Their results also suggested that even though angiosperm and gymnosperm are distantly related groups of plant species, their reproductive growth is probably controlled by a similar group of homeotic genes.

Flower development is an important process during the life history of a plant. It not only fulfils the role as a reproductive organ for the plant itself, but also has a significant impact on the environment and human beings. A thorough understanding of the genetic control of flower development is necessary if any attempt is going to be made to improve the performances and productivities of certain plant species using genetic manipulation techniques.

Pollen, as a major product from flower development is a carrier of genes, nutrients, energy, and allergenic proteins. Hence, once pollen is released into the environment, there are many considerations to take into account.

With the introduction of genetic engineering techniques into operational forestry programs, the production of genetically altered trees through asexual transfer of genes has been a focus in most research. Genes governing commercially useful traits such as wood quality have been the major targets (Jouanin *et al*., 1993) (Whentten and Sederoff, 1991). But Strauss *et al* (1995) pointed out that the incorporation of transgenic trees into operational forestry programs requires many additional steps before commercial use is possible. Apart from the continuing need to develop efficient gene-transfer methods for commercially desirable genotypes, the major constraints to the use of transgenic trees are ecological safety and regulatory approval. Safety considerations are of paramount importance, as most genetic engineering of crops results in traits such as herbicide resistance traits, insect resistance traits, or viral resistance, and these all could confer a fitness advantage to a wild plant. So Strauss *et al* (1995) pointed out that if the transgenic plants were introduced into the environment they could enhance weediness through the release of these transgenes into the gene pool of wild weed species. They also pointed out that transgenic plants could hybridise through pollination with their wild relatives.

Ellstrand *et al* (1990), Rissler *et al* (1993) and Tiedje *et al* (1989) stated that these two problems could result in the production of offspring with increased invasiveness and such secondary impacts as loss of biological diversity through displacement of native species. Bearing this ecological consideration in mind, Strauss *et al* (1995) concluded that containment of transgenes inserted into genetically engineered forest trees would probably be necessary before most commercial uses were possible, and the containment of transgenes could be achieved through engineering of male sterile trees.

In addition to gene containment, engineering of complete or male sterility may stimulate faster wood production. Substantial energy and nutrients were reported to be committed to reproductive development in trees (Ledig 1986). Fielding (1960) calculated that the energy invested in cones and pollen of radiata pine (*Pinus radiata*) was equivalent to a 16% reduction of mean annual increment in wood growth. Cremer (1992) reported that both male cones and foliage fascicles of *Pinus radiata* originate as short-shoot initials, each male cone is produced at the expense of one fascicle of foliage. On average, about 13% of the potential foliage was diverted to the production of male flowers. He did not find that the stem growth per tree within a stand, during or shortly after the main growth of male cones varied with the abundance of male cones on those trees. However he did find that in dense stands male cone production caused a nutrient stress on vegetative growth. This effect became even more severe when the site was nutritionally poor, because the limited nutrient capital had to be shared amongst cones and vegetative parts. Dick *et al* (1990) also reported that branches bearing male cones had 33% fewer needles compared with equivalent vegetative branches in *Pinus contorta*. They found a significant reduction in the dry weight per needle on the parent shoots of reproductive branch units, compared to vegetative branch units, suggesting that male cones were a larger drain on the parent shoot of reproductive branch units than the vegetative shoots were on vegetative branch units. They also reported that early in the growing season, reproductive branch units (branches bearing male cones) allocated approximately 45-65% of the total dry weight of the current growth into male cones, indicating their importance as photosynthate sinks (Dicks *et al.*, 1990). Based on these facts, engineering male sterility would be expected to increase vegetative growth, i.e. wood production, in these conifers.

Another justification for generating male sterility is to remove a source of pollen derived allergens from the air. For example, in Japan many people were reported to suffer from

allergies induced by the most commonly planted forest tree, the conifer sugi (*Cryptomeria japonica*) (Ishizaki *et al.*, 1987). Similar complaints were also reported from some local Maori populations who live near the large radiata pine forest in the central part of the North Island in New Zealand. One of every six families was reported to have suffered from some kind of allergic diseases, possibly caused by the large amount of pine pollen release in that area (Fountain and Cornford, 1991).

Concerns over genetic pollution of native populations by bred varieties of trees through pollen release were also reviewed by Strauss *et al* (1995). They stressed that "large influxes of foreign pollen or seeds might undermine the genetic integrity, diversity, or adaptedness of native populations, when large areas of markedly different genotypes, such as different provenances, exotic species, or novel hybrids were installed close to small native stands." They pointed out that in such cases, engineered sterility would greatly reduce the impacts of intensively bred tree plantations on nearby stands.

The last, but not least contribution of engineering male sterility worthy of mentioning is to facilitate hybrid breeding. Studies on organ/tissue-specific genes have led to some important findings on the genetic mechanism of the anther (male floral organ) development and male sterility. For instance, a number of meiosis-specific proteins were identified in lily plant. These included a unique endonuclease (Howell and Stern, 1971), DNA-unwinding protein (Hotta and Stern, 1978), DNA reassociation protein (Hotta and Stern, 1979) and a RecA-like protein which specifically occurred during meiosis in microsporogenesis (Hotta *et al.*, 1985). There were also numerous examples of nonallelic isozymes expressed in anthers: ADP-glucose pyrophosphorylase (Bryce and Nelson, 1979), β-galactosidase (Frova *et al.*, 1987), α- (Ludwig *et al.*, 1988) and β-tubulins (Hussey *et al.*, 1988).

The most important contribution of studies on the anther-specific genes is in providing a better understanding of the genetic mechanism of male sterility in plants. This knowledge provides a number of ways of generating male sterile plants. Since many types of natural male sterility result from errors in tapetal development or physiology (Kaul, 1988), it was realised by most groups working on this problem that the engineered destruction of the tapetum offered the most direct route to male sterility. Thus tapetum-specific promoters, the key components of the first generation of such systems were developed by Goldberg (1988). Mariani *et al* (1990) demonstrated that expression of RNAases T1 or Barnase,

under the transcriptional control of the tapetum-specific promoter TA29 from tobacco, resulted in destruction of the tapetum and male sterility due to a failure to produce pollen grains. Importantly, female fertility was unaffected. Tapetum-specific expression of the gene for an Endo-β-1,3-glucanase causing male sterility in transgenic tobacco was reported by Tsuchiya, *et al* (1995). They reported that" the introduced gene for Endo-β-1,3-endoglucanase under the control of the *Osgb6* promoter (anther tapetum-specific promoter) caused digestion of the callose wall at the beginning of the tetrad stage, a time that was just a little earlier than the time at which endogenous glucanase activity normally appeared." (Tsuchiya *et al.*, 1995). These results demonstrated that premature dissolution of the callose wall in pollen tetrads caused male sterility and suggested that the time at which tapetal glucanase activity appear was critical for the normal development of microspores.

The isolation of tapetum-specific genes is important for understanding the function of the tapetum and the transport of the tapetal products from this tissue to the pollen exine. In addition to the *TA*29 gene of tobacco (Koltunow *et al.*, 1990), many genes were found to be expressed specifically in the developing anthers and some of them have been shown to be tapetum-specific by *in situ* hybridization experiments. These include, clone 108 gene of tomato (Smith *et al.*, 1990), *TAP*1 gene of *Antirrhinum majus* (Nacken *et al.*, 1991), *BA*112 gene (Shen *et al.*, 1992) and *A*9 gene (Scott, *et al.*, 1991) of *Brassica napus*, *MFS* gene of maize (Wright *et al.*, 1993) and *Osg*6B gene of rice (Tsuchiya *et al*,. 1994).

Apart from these anther-specific genes, some pollen-specific genes have also been identified. For example, a pollen-specific sequence, *NeIF-4A8* was isolated from a cDNA library from mature pollen of *Nicotiana tabacum* cv. Samsun by Brander *et al* (1995). They reported that *NeIF-4A8* was a full-length cDNA whose deduced amino acid sequence exhibited high homology to the eucaryotic translation initiation factor eIF-4A from mouse, *Drosophila* and tobacco. eIF-4A was an RNA helicase which belongs to the supergene family of DEAD-box proteins reported by Schmid, and Linder (1992). By doing a Northern blot analysis with a gene-specific probe, Brander *et al* (1995) detected the strict microspore-specific expression of *NeIF-4A8* starting at mitosis. They suggested that *NeIF-4A8* was a prime candidate for mediating translational control in the developing male gametophyte. Another pollen-specific cDNA clone, *Zmc*13 was isolated from a cDNA library constructed to poly(A) RNA from mature maize pollen by Hanson *et al*

(1989). *In situ* hybridisation using RNA probes in their study showed that the mRNA of *Zmc*13 was located in the cytoplasm of the vegetative cell of the pollen grain and after the germination of pollen the mRNA was distributed throughout the pollen tube cytoplasm. Their results suggested that this mRNA was most likely a product of transcription of the vegetative cell nucleus and indicated that *Zmc*13 gene was more likely involved in the pollen tube growth rather than the development of the sperm cell (Hanson *et al.*, 1989).

With the progress of studies on homeotic genes, a MADS-box gene, *AP*3 from *Arabidopsis thaliana* was identified as an organ specific gene, controlling the stamen and petal development (Jack et al,. 1992). Day *et al* (1995) used the *AP3* promoter developed by Irish and Yamamoto (1995) to drive the expression of the diphtheria toxin A chain coding sequence DTA. DTA is an inhibitor of protein synthesis, which can cause internucleosomal fragmentation of DNA (Kochi and Collier 1993; Martin, 1993). The *AP*3 promoters lead to expression in second and third whorl (petal and stamen) primordia in *Arabidopsis thaliana* and *Nicotiana tabacum* floral meristems. In both species the ablated flowers failed to develop petals and stamens. Even though there was a weak expression in non-target organs (carpels), carpel and sepal development in both species appeared to be largely unaffected. Day *et al* (1995) proposed that spatial signals defining the organ primordia were established before *AP3* was expressed in the floral meristem (before the formation of petals and stamens). Their result also provided another strategy of generating male sterility.

To identify and characterise these anther-specific and pollen-specific genes and use part of them as male-specific promoters to express a cytotoxic gene was the major strategy for genetically ablating floral tissues. This cytotoxic gene only expressed in the particular male tissues under the control of male organ-specific promoters and killed cells of that tissue, causing male sterility. The disadvantage of this strategy is that it usually only acts at the late developmental stages of the floral organs, unlike some of the homeotic genes which have the potential to act early in reproductive development, such as *LEAFY* or *AP1*.

Another concern of this method raised by Strauss *et al* (1995) was that the inserted cytotoxins were likely to cause gene shutdown or down-regulation caused by methylation or position effects. They suggested that insertion of more than one construct using different promoters and coding regions to avoid cosuppression would enhance stability.

Another major strategy to generate male sterility is disruption of the expression of genes essential for fertility. Strauss *et al* (1995) also reviewed latest achievements of using antisense and sense suppression methods to generate sterility. These methods depended on identifying expressed genes needed for development of reproductive organs, but did not depend on the use of promoters that function exclusively in floral tissues. They pointed out that the transforming sequences only needed to match a portion of the target gene. Antisense RNA acted either by reducing mRNA translation of the target gene or by increasing mRNA degradation of the target gene. This strategy was reviewed by Kooter (1993) and Mol *et al* (1994). Sense suppression was associated with the introduction of duplicate copies of either a native gene or transgene. They could reduce expression of the original gene, the newly introduced gene or both. This strategy was reviewed by Flavell (1994) and Jorgensen (1992).

Antisense RNAs targeted against several floral genes have been used to generate sterile plants. For example, when an antisense version of *TAG1* (the tomato homologue of *AG* ) driven by the CaMV 35S promoter was introduced into tomato, plants with aberrant male- and female-sterile flowers were obtained. There were no detectable effects on vegetative organs (Pnueli *et al*., 1994). Sense suppression has also been used to induce sterility and interfere with floral development. Angenent *et al* (1993) reported that expression of a *Petunia* floral homeotic gene that contained a MADS-box, *fbp1*, was inhibited when expression of sense transcripts was driven by the CaMV 35S promoter. No *fbp1* mRNA was detected in developing flowers, indicating that suppression was complete and flowers were male- and female-sterile.

To conclude these strategies of generating male-sterility in plant species, we see that it is absolutely essential to identify expressed genes needed for development of reproductive tissues/organs. Thus the isolation and identification of DNA sequences related to genes needed for development of reproductive tissues/organs from *Pinus radiata* are the starting point of the endeavour to generate male sterility in this species.

As reviewed before, MADS-box genes are found to be responsible for the development of reproductive tissue/organs in almost every species studied so far, including both gymnosperm and angiosperm species, suggesting the conservative nature of this group of genes. It has also been reviewed (Yanofsky, 1995) that genes containing a MADS-box

region are involved in the development of both floral meristem and floral organs. Isolating DNA sequences containing MADS-box regions could lead us to locate a number of different MADS-box genes from *Pinus radiata*. This procedure could probably provide us with information on genes controlling different stages of developments of the floral tissues/organs in *Pinus radiata*.

In order to examine the possible existence of MADS-box genes in *Pinus radiata*, a search for DNA sequences related to the *Arabidopsis AGAMOUS* gene in *Pinus radiata* was made by Southern blot analysis.

In order to isolate DNA sequences containing MADS-box a PCR technique was developed using a set of degenerate primers targeting the conserved regions of the MADS-box to isolate DNA sequences related to these MADS-box genes from *Pinus radiata*.

# 4. 2 MATERIALS AND METHODS

## 4. 2. 1. MEDIA, BUFFERS AND SOLUTIONS

### 4.2.1.1. LB Media

LB media contained (g/l): tryptone (Difco), 10.0; yeast extract (Difco), 5.0; NaCl, 5.0. The pH was adjusted to 7.0 prior to autoclaving. For solid media, agar (Davis) was added to 15.0 g/l. Where needed, ampicillin was supplemented at a concentration of 100 μg/ml, isopropylthio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside in dimethylformamide (X-gal) were both supplemented at a concentration of 40 μg/ml.

### 4.2.1.2. 2 x YT Media

2 x YT media contained (g/l) : tryptone (Difco), 16.0; yeast extract (Difco), 10.0; NaCl, 5.0. 0.5 ml of 5 M NaOH was added to pH 7.4 prior to autoclaving. This media did not need to be diluted before use.

### 4.2.1.3. Top Agarose

Top agarose contained (g/l): tryptone (Difco), 10.0; NaCl, 8.0; agarose (BDH), 8.0. This was cooled to 45-50$^0$C following autoclaving and supplemented with $MgSO_4$ to 10 mM.

### 4.2.1.4. 1 x TBE Buffer

1 x TBE buffer contained 89 mM Tris-HCl, 2.5 mM $Na_2EDTA$, and 89 mM boric acid, pH 8.3.

### 4.2.1.5. STET Buffer

Stet buffer contained 8% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM $Na_2EDTA$ (pH 8.0) and 50 mM Tris-HCl (pH 8.0).

### 4.2.1.6. SDS Loading Buffer

SDS Loading buffer contained 1% (w/v) sodium dodecyl sulphate (SDS), 0.02% (w/v) bromophenol blue, 20% (w/v) sucrose, and 5 mM $Na_2EDTA$ (pH 8.0).

4.2.1.7. TE Buffer and TAE Electrophoresis Buffer

TE buffer (10 mM Tris-HCl/1 mM $Na_2EDTA$ or 10 mM Tris-HCl/0. 1 mM $Na_2EDTA$) was prepared to the required concentration from 1 M Tris-HCl (pH 8.0) and 250 mM $Na_2EDTA$ (pH 8.0) stock solutions.

TAE electrophoresis buffer [40 mM Tris-HCl, 20 mM glacial acetic acid and 2 mM $Na_2EDTA$, (pH 8.2)]

4.2.1.8. 20 x SSC and 3 x SSC

20 x SSC contained 3 M NaCl and 0.3 M sodium citrate.

3 x SSC was prepared by appropriate dilution of 20 x SSC.

4.2.1.9. Prehybridisation Buffer

This hybridisation buffer contained (per 200 ml): 30 ml 20 x SSC; 4 ml 0.02% 50 x Denhardts [Ficoll 0.5 g, polyvinylpyrrolidone (PVP) 0.5 g, and albumin bovine (BSA) 0.5 g in 50 ml MilliQ water, stored at $-20^0$C]; 6 ml 10% SDS; 1 ml ssDNA (Salmon sperm DNA 10mg/ml) and 159 ml MilliQ water. The final concentration of each component in this prehybridisation buffer (200 ml) is 3 x SSC, 0.02% Denhardt's, 0.5% SDS and 50µg/ml ssDNA.

4.2.1.10. TES Buffer (10/1/100)

TES (10/1/100) buffer contained 10 mM Tris-HCl (pH 8.0), 1 mM $Na_2EDTA$ (pH 8.0) and 100 mM NaCl.

4.2.1.11. Tris-Equilibrated Phenol

Tris-Equilibrated Phenol was prepared by melting solid phenol at 50°C. Hydroxyquinoline was added to a final concentration of 0.1% (w/v). An equal volume of 1 M Tris-HCl (pH 8.0) was added at room temperature and stirred for 15 min. The phenolic phase was retained and repeatedly washed with 1 M Tris-HCl (pH 8.0), until the pH of the phenolic phase was > 7.8. After equilibration the phenolic phase was retained and washed 2-3 times with 100 mM Tris-HCl (pH 8.0). The equilibrated phenol solution was stored under 100 mM Tris-HCl (pH 8.0) in a brown bottle at 4°C.

4.2.1.12. Acrylamide mix

Acrylamide mix contained (g/l): urea, 480 g; acrylamide, 57 g; bisacrylamide, 3 g. This mix was made up to < 900 ml and deionised with Amberlite MB-3 (Sigma), then filtered through a sintered glass funnel (porosity 1), 100 ml of 10 x sequencing TBE

buffer (section 4.2.1.14) was then added and the volume made up to 1 litre with MilliQ water.

4.2.1.13. DNase free RNaseA

DNase free RNaseA was prepared from RNaseA at 10 mg/ml in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl, heated to 100°C for 15 min, allowed to cool slowly to room temperature, dispensed into aliquots and stored at -20°C.

4.2.1.14. 10 x Sequencing TBE Buffer

10 x Sequencing TBE buffer contained (g/l): Tris, 162 g; $Na_2EDTA$, 9.5 g; boric acid, 27.5 g. For running sequencing gels this buffer was diluted 10 x with MilliQ water.

4.2.1.15. 10 x PCR amplification buffer

10 x PCR amplification buffer contained 500 mM KCl, 100 mM Tris HCl (PH 8.3), 15 mM $MgCl_2$ and 0.1% gelatine. (Sambrook *et al.*, 1989)

4.2.1.16. CTAB DNA extraction buffer

CTAB DNA extraction buffer contained 1% PEG 8000, 100 mM Tris-HCl, 1.4 M NaCl, 20mM EDTA and 2% Cetyltrimethyl ammonium bromide (CTAB). The pH was adjusted to 9.5 prior to autoclaving (Ben Sutton at BC Research, Vancouver, Canada, personal communication).

4.2.1.17. *AGAMOUS (AG)* plasmid DNA

*AG* plasmid DNA was cloned by Yanofsky *et al* (1990). The *AG* cDNA insert was cloned into the *Eco* RI site of pGEM7Zf(+) ($Amp^r$)

4.2.1.18. *LEAFY (LFY)* plasmid DNA

*LFY* plasmid DNA was cloned by Weigel *et al* (1992). The *LFY* cDNA insert was cloned into the site between *Bam* HI and *Kpn* I restriction site of pBluescript KS+ ($Amp^r$).

4.2.1.19. DNA molecular weight marker λ DNA digested with *Eco* RI + *Hind* III (Boehringer Mannheim)

Fragment sizes: 21 226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564 base pairs.

4.2.1.20. DNA molecular weight marker pBR322 DNA digested with *Hinf* I (New England Biolabs)

Fragment sizes : 1631, 517, 506, 396, 344, 298, 221, 154, 72.

4.2.1.21. DNA molecular weight marker: 1 kb DNA ladder (GIBCO BRL)

Fragment sizes : 12 216, 11 198, 10 180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, (517, 506, 396, 344, 298, 221, 154, 72 *Hinf*I fragments of the vector) base pairs.

4.2.1.22. M13mp18 RF DNA From *E.coli* (Boehringer Mannheim GmbH)

The multiple restriction sites are:

```
                                                        Sal    I
                                                        Acc    I
          Eco RI          Kpn   I     Bam HI          Hind   I
5'..GATTACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAG
                Sac   I       Sma   I      Xba   I          Pst   I
                              Xma   I
Sph   I
GCATGCAAGCTTGGCG...3'
      Hind III
```

## 4.2.2. DNA ISOLATION

4.2.2.1. Miniprep DNA Isolation from needle fascicle tissue of *Pinus radiata* and the leaf tissue of *Arabidopsis thaliana.*

DNA was extracted from needle fascicle tissue of radiata pine clone 880-606 using the method of Sutton (personal communication). DNA was extracted from the leaf tissue of *Arabidopsis thaliana* using the same method. In a pre-cooled mortar and pestle 0.5-1 g of freeze dried pine needle fascicle tissue or leaf tissue of *Arabidopsis* was ground to a fine powder under liquid nitrogen and then suspended in 850 µl CTAB extraction buffer (section 4.2.1.16.) and 25 µl/ml 10% SDS. The solution was mixed by inversion to form an emulsion. The mixed solution was incubated at $65^0$C for 15 minutes. 500 µl chloroform was added to this solution. It was mixed by inversion and centrifuged for 5 minutes at 15 000 g. The top aqueous layer of this centrifuged solution was transferred to a fresh eppendorf tube. 1/10 (50 µl) 3 M ammonium acetate was added to this supernatant and mixed by inversion. Then isopropanol/ethanol DNA precipitation (section 4.2.3.2.) was carried out. DNA was then resuspended in 500 µl of TE buffer (section 4.2.1.7.) and quantitated (section 4.2.4.).

4.2.2.2. Plasmid DNA Isolation by the Rapid Boiling Method

Cells from 1.5 ml of an overnight E. *coli* LB broth culture (section 4.2.1.1.), containing appropriate antibiotics and shaken at either 30°C or 37°C, were pelleted by a 1 minute centrifugation in a 1.5 ml microcentrifuge tube. The supernatant was drained and the pellet was resuspended in 350 µl of STET buffer (section 4.2.1.5.). Lysozyme (25 µl of a 10 mg/ml solution) was added and the tube was placed in a boiling water bath for 40 seconds. The tube was then centrifuged for 10 min in a microcentrifuge and the resulting gelatinous pellet was removed with a sterile tooth pick. The DNA was precipitated by the addition of an equal volume of isopropanol. The contents were mixed by inversion and the tube was allowed to stand on ice for 10-20 min. The plasmid DNA was pelleted by centrifugation for 5 min in a microcentrifuge. The plasmid DNA pellet was washed once with 95% ethanol at room temperature, dried under a vacuum for 15 to 30 min prior to resuspension in 50 µl of MilliQ water or TE (10/0.1) (section 4.2.1.7.) and quantitated (section 4. 2.4.). This method was based on that of Holmes and Quigley (1981).

## 4.2.3. DNA PURIFICATION

### 4.2.3.1. Purification of DNA by phenol/chloroform extraction

DNA samples were extracted with equal volumes of Tris-equilibrated phenol (section 4.2.1.11.) and chloroform, centrifuged ( 15 000 g), and the aqueous phase re-extracted until a clear interface between the aqueous and organic phases was obtained. Samples were then extracted once with two volumes of chloroform. The DNA was then precipitated by ethanol/isopropanol precipitation as described in section 4.2.3.2. This method was based on that of Sambrook *et al.* (1989).

### 4.2.3.2. Precipitation of DNA with ethanol or isopropanol

One tenth volume of 3 M sodium acetate and either 2.5 volumes of 95% ethanol, or 0.6 volumes of isopropanol, were added to a tube containing DNA and kept on ice for at least 15 minutes after which time the DNA was pelleted by centrifugation at 15 000 g for 5-10 minutes. The pellet was washed once with 70% ethanol and dried under vacuum until the alcohol had evaporated, before resuspension in MilliQ water or TE (Section 4.2.1.7.). This method was based on that of Sambrook *et al.* (1989).

As noted above, 0.6 volumes of isopropanol was sometimes used in place of ethanol, however, ethanol was preferred as it is more volatile and solutes (e.g. NaCl) are less easily coprecipitated, thereby minimising coprecipitation of components that may prevent re-dissolution of the DNA.

## 4.2.4. DNA QUANTIFICATION

DNA was quantified by three methods. A spectrophotometric method (section 4.2.4.1.) was used to check the purity of DNA sample. A fluorometric method (section 4.2.4.2.) usually gave a consistent and reliable result, so this method was used routinely for DNA quantification. For impure DNA samples of very low concentration, DNA was quantified by comparing the intensity of ethidium bromide fluorescence of the sample DNA with that of a series of standard DNA concentration markers on an agrose gel (section 4.2.4.3.).

### 4.2.4.1. Spectrophotometric Determination of DNA Concentration

Concentrated DNA solutions were diluted appropriately and the absorbance of the solutions in quartz cuvettes with a 1 cm light path was determined at both 260 nm and 280 nm. The reading at 260 nm allowed calculation of the concentration of nucleic acid present in the sample since an OD of 1 corresponds to approximately 50 μg/ml double stranded DNA. The ratio of readings at 260 nm and 280 nm ($OD_{260}/OD_{280}$) was used as an estimate of the DNA purity. Pure DNA has an $OD_{260}/OD_{280}$ value of 1.8.

### 4.2.4.2. Fluorometric quantitation of DNA

For most DNA samples, DNA was quantitated on a Hoefer Scientific TKO 100 Fluorometer. This method was suitable for quantitating down to 10 ng/μl and only 2 μl of DNA sample was needed for quantitation. DNA was quantitated in a dye solution containing 1 x TNE buffer (10 mM Tris-HCl, 1mM $Na_2EDTA$ and 100 mM NaCl, pH 7.4) and 0.1 μg/ml Hoechst 33258. The scale of the fluorometer was set to 100 using 2 μl of 100 μg/ml calf thymus DNA added to 2 ml of the dye solution. Once the scale was reliably calibrated, 2 μl of sample DNA was added to 2 ml of the dye solution and the resulting figure recorded as a concentration of ng/μl for the sample DNA solution.

### 4.2.4.3. Minigel method for determination of DNA concentration

A sample from the DNA solution of interest was separated by electrophoresis through an agarose gel (section 4.2.6.) together with a series of standard DNA solutions of known concentration. After the bromophenol blue dye front had migrated at least half way into the gel, the DNA was stained with ethidium bromide and photographed (section 4.2.6.). The intensity of fluorescence of the unknown DNA sample was then compared to that of the known DNA standards.

### 4.2.5. RESTRICTION ENDONUCLEASE DIGESTION OF DNA

Restriction endonuclease digests were carried out in accordance with the manufacturers recommendations or with the manufacturers buffer supplied with the enzyme. DNA to be digested was quantitated (section 4.2.4.) and an excess of enzyme used to digest the DNA. Digestions of plasmid and phage λ DNA were performed at the recommended temperature for 1 hour and stored on ice or at -20°C, while an aliquot was checked on an agarose gel (section 4.2.6.) to ensure completeness of digestion. In the event that a digestion was incomplete fresh enzyme was added and the mixture was incubated a further 1 hour. Quantities of restriction endonucleases added were kept within suitable ranges (< 10% of the total volume) to avoid non-specific cleavage by the enzyme. In the event that the DNA was still not digested to completion, the DNA stock was further purified, by ethanol/isopropanol precipitation (section 4.2.3.2.), and the digest repeated. Once complete, digestion was stopped by addition of 1/5 volume SDS loading buffer (section 4.2.1.6.). Digestion of pine genomic DNA in a total volume of 500 µl was performed in an identical fashion to the digestion of plasmid and phage λ DNA except digestion times were increased to a minimum of 3 hours and a maximum of overnight incubation.

### 4.2.6. AGAROSE GEL ELECTROPHORESIS OF DNA

Horizontal agarose gel electrophoresis was performed either in a Mini-gel apparatus for 1-2 h or in a Biorad DNA Sub-Cell (150 x 200 mm gel bed) overnight. Agarose concentrations varied from 0.8% to 3%. The appropriate quantity of agarose was added to 200 ml of TAE electrophoresis buffer (section 4.2.1.6.) and the agarose was melted in a microwave. After cooling to 50°C in a water bath, the gel was poured and allowed to set. DNA samples (with addition of 1/5 volume SDS loading buffer, section 4.2.1.6) were loaded and the fragments were separated by electrophoresis at 80 V to 120 V (Minigels) or 30 V (Biorad Sub Cell). After electrophoresis, gels were stained with ethidium bromide, typically for 15 minutes, washed with MilliQ water, visualised on a UV transilluminator and photographed on Polaroid type 667 film. DNA fragment sizes (in kilobases, kb) were determined, after agarose gel electrophoresis, by measuring the distance a fragment had migrated from the well in the gel. The molecular weight was then calculated by interpolation from a plot of the distance migrated in the same gel by

suitable size (molecular weight) markers, against the logarithm of the molecular weight (kb or bp) of the size markers.

### 4.2.7. RECOVERY OF DNA FROM AGAROSE GELS

DNA was recovered from SeaPlaque agarose gels [0.7% to 1.5% in TAE electrophoresis buffer (section 4.2.1.6.)] by GLASS MAX™ DNA Isolation Spin Cartridge System and Polyester Filter System. After gel electrophoresis to separate DNA fragments, the DNA fragment(s) of interest were visualised with a long wave UV lamp, excised from the gel with the minimum amount of excess agarose and placed in 1.5 ml microcentrifuge tubes. Then DNA samples were either isolated through the Spin Cartridge System or Polyester Filter System.

4.2.7.1. Glassmax™ DNA isolation spin cartridge system.

The excised agarose gel was weighed and 0.45 ml Binding Solution (6 M Sodium Iodide NaI) was added into each microcentrifuge tube with 0.1 g of agarose gel. The gel slices in Binding Solution were heated at 55$^0$C until the agrose was fully dissolved. Up to 550 µl of DNA/Nal solution was added to the Glass MAX Spin Cartridge and was capped. The spin cartridge was centrifuged at 15,000 g for 20 seconds. The tube was emptied and the solution was saved until recovery of the DNA was confirmed. 0.4 ml of cold (4$^0$C) wash buffer (wash buffer contained 4 ml of wash buffer concentrate, 71 ml of distilled water and 85 ml of absolute ethanol, stored at 4$^0$C) was added to the spin cartridge. The spin cartridge was centrifuged at 15,000 g for 20 seconds and the tube was emptied. This wash step was repeated two additional times. After the final wash buffer was removed from the tube, the spin cartridge tube was centrifuged at 15,000 g for 1 minute. The spin cartridge insert was transferred into a Sample Recovery Tube. 40 µl of the TE (section 4.2.1.7.) buffer that was preheated to 65°C was added to the spin cartridge insert and then the spin cartridge unit was centrifuged at 15,000 g for 20 seconds to elute the DNA. DNA was quantitated (section 4.2.4.3.) and stored at 4$^0$C (GIBCO BRL LIFE TECHNOLOGIES, INC.).

4.2.7.2. Polyester filter DNA isolation method (Struhl, 1994)

The cap of a small (0.5-ml) microcentrifuge tube was removed, and a hole was made in the tip of the small microcentrifuge. A piece of polyester filter was inserted into the small microcentrifuge and the small tube was put into a larger (1.5-ml) microcentrifuge

tube. The gel slice was positioned on the polyester filter inside the small tube. The microcentrifuge tube was centrifuged at 15, 000 g for 5 minutes. Clean DNA was filtered through the polyester into the large tube, leaving agarose debris trapped in the filter material. The DNA was then precipitated by ethanol/isopropanol method (section 4.2.3.2.).

## 4.2. 8. SOUTHERN BLOTTING AND HYBRIDISATION

### 4.2.8.1. Southern (Capillary) Blotting

DNA to be transferred to the Nylon membrane was separated by overnight gel electrophoresis, stained, visualised and photographed as described in section 4.2.6. The gel was placed 250 mM HCl and gently agitated for 2 x 15 min for two times (depurination treatment). The HCl was poured off and the gel was immersed in denaturing solution (500 mM NaOH, 500 mM NaCl), with gentle agitation, for 30 minutes. The denaturing solution was drained and the gel was immersed in neutralising solution (500 mM Tris (pH 7.4), 2 M NaCl), with gentle agitation, for 15 min. The gel was finally washed for 2 min in 3 x SSC (section 4.2.1.8.). The gel was washed in two changes of MilliQ water between all changes of solutions. While the gel was being treated, a plastic trough with wells at each end was prepared by placing two sheets of Whatman 3MM chromatography paper soaked in 20 x SSC (section 4.2.1.8.) in the trough such that the ends of the paper projected into the wells. The wells were then filled with 20 x SSC to just below the horizontal surface of the paper between the wells. A sheet of Gladwrap was placed over the trough and pressed flat. A grid 2 mm less than the gel size was marked on the Gladwrap and removed. The treated gel was placed, inverted, over the grid such that the edges of the gel overlapped the edges of the grid. A piece of nylon membrane (Hybond-N, Amersham), cut to 2 mm greater than the gel size and pre-soaked in 3 x SSC, was placed over the gel ensuring that no air bubbles were present. Two pieces of Whatman 3MM chromatography paper, cut 2 mm less than the gel size and pre-soaked in 3 x SSC, were placed over the membrane. Two identically sized pieces of Whatman 3MM chromatography paper (unsoaked) were placed upon the two soaked pieces of 3MM paper. A stack of paper towels approximately 50 mm deep was placed upon the chromatography paper, followed by a flat plastic tray and a weight sufficient to keep the entire stack flat. After overnight

transfer, the apparatus was disassembled and the membrane was washed for 5 minutes in 3 x SSC, then baked under vacuum at $80^0$C for 2 hours. This method was based on the method of Southern (1975).

4.2.8.2. Preparation of [$\alpha$-$^{32}$P]dCTP-labelled probe with the Ready-To-Go DNA Labelling Kit. (Random labelling method).

DNA to be labelled (25-50 ng), in a 45 µl volume, was denatured in a boiling water bath for 3 minutes then immediately placed on ice for 2 minutes to cool. The denatured DNA solution was then added to the tube containing the Ready-To-Go (Pharmacia Biotech.) reagent mix, 3-5 µl of [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol) was added, and if needed, MilliQ water to a final volume of 50 µl. The reaction was mixed by gently tapping the tube, spun briefly in a microcentrifuge and incubated at $37^0$C for 15-60 minutes. Unincorporated nucleotides were separated from labelled DNA on a minispin Sephadex G-50 column (section 4.2.8.3.) equilibrated with TES (section 4.2.1.10.).

4.2.8.3. Separation of Unincorporated Nucleotides by Minispin Column Chromatography

Minispin columns were constructed by plugging the bottom of a 1 ml plastic, disposable, Terumo Tuberculin syringe with siliconised glass wool. The syringe was filled with Sephadex G-50 resin, equilibrated in TES (10/1/100) (section 4.2.1.10.). Additional resin was added until the syringe was full to the 1 ml mark. The end of the syringe was inserted into the perforated cap of a 1.5 ml microcentrifuge tube. The assembly was inserted into a disposable plastic tube and centrifuged at speed 3 in a BTL bench centrifuge (approximately 1 500 g) for 4 minutes at room temperature in a swinging bucket rotor (all subsequent centrifugation steps described were also at the same speed and duration). Additional resin was added until the volume of resin in the syringe, after centrifugation, was unchanged at approximately 0.9 ml, 100 µl of TES (10/1/100) (section 4.2.1.10.) was then added to the column, which was recentrifuged, this step was repeated twice. The radiolabelled DNA was added to the column in 100 µl of TES (10/1/100) and recentrifuged into an empty 1.5 ml microcentrifuge tube.

4.2.8.4. Hybridisation of Probe DNA to Southern Blots

The Southern blot (section 4.2.8.1.) to be probed was prehybridised for at least 2 hours at 60°C (unless otherwise indicated) in prehybridisation buffer (section 4.2.1.9.) in a

sealed glass tube. After prehybridisation, all but approximately 5 ml of the hybridisation buffer was poured off, and [$\alpha$-$^{32}$P]dCTP-labelled probe which had been heated to 100$^0$C was then added (section 4.2.8.2.).

After overnight hybridisation at 60°C (unless otherwise indicated), the filter was removed and washed with 3 x SSC, 0.2% SDS solution (section 4.2.1.8.). The washed filter was then wrapped in Gladwrap while still damp, and exposed, in the presence of a Cronex intensifying screen, to a sheet of Fuji Medical X-ray film in a X-ray cassette. After exposure for a suitable period of time at -70°C (from 3 days -7 days), the film was developed.

4.2.8.5. Stripping Hybridised DNA off Southern Blots

A boiling solution of 0.1% sodium dodecyl sulphate (SDS) was poured over the filter to be stripped, and the filter was gently shaken in this solution 3 x 20 minutes while the solution cooled to room temperature. The filter was then checked by autoradiography overnight (as outlined in section 4.2.8.4.) to ensure that stripping of the filter had occurred. If stripping was incomplete this process was repeated.

## 4.2.9. AMPLIFICATION OF DNA BY THE POLYMERASE CHAIN REACTION (PCR)

4.2.9.1. Primer designing for the amplification of the genomic DNA of *Pinus radiata* by PCR

The PCR technique was applied to isolate the MADS-box DNA sequences from the genomic DNA of *Pinus radiata*. Degenerate primers were designed based on the conserved region of the MADS-box from the *AGAMOUS* gene of *Arabidopsis* (Yanofsky *et al.*, 1990). The amino acid sequence of the MADS-box domain of *Arabidopsis AGAMOUS* is shown here:

GRGKIEIKRIENTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALIVFSSRGRLYEY

Conserved regions of the MADS-box domain are underlined.

The forward primer is 5'-CGGAATTCATTA/CGICAG/AGIACIT-3' coding for RQVTF, the reverse primer is 5'-GCTCTAGATC/TIGCA/GTCA/GCAIAA/GIA-3' (complementary to the amino-acid sequence VLCDAE). (A/C: either A or C, G/A: either G or A, C/T: either C or T, and A/G: either A or G. I: inosine, it is a substitution for nucleotide A, G, T, and C). For the convenience of cloning, restriction sites *Eco* RI

(underlined) and *Xba* I (underlined) were attached at the ends of the primers. The PCR product is expected to be 8 + 78 + 8 = 94 bp, providing that the MADS gene in *Pinus radiata* is 100% conserved.

4.2.9.2. Conditions for the amplification of the genomic DNA of *Pinus radiata* by PCR

For n PCR reactions a cocktail for n+l reactions was prepared on ice. A cocktail for 1 reaction contained: 10 µl of 10 x *Taq* PCR buffer (section 4.2.1.15.); 6.4 µl of 1.25 mM dNTPs; 4-8 µl each of 20 µM "forward" primer and "reverse" primer (section 4.2.9.1.); 2 units of Taq DNA polymerase (Boehringer Mannheiim GmbH); and MilliQ water to 100 µl. The cocktail was aliquoted out into 100 µl quantities in 0.2 ml strip tubes for use in a Corbett FTS-960 thermal cycler. A quantity of 1 µg DNA expected to contain the sequence to be amplified, in a volume of 10 µl, was added to the appropriate tube and mixed gently. A negative control containing water only was always included in each PCR run, this was prepared as the last reaction in each set. The reaction vessels were placed in the appropriate thermal cycler preheated to $94^0$C and after an initial 5 minutes melt at 94°C subjected to 20 cycles of 94°C for 45 sec, 37°C for 45 sec and 72°C for 60 sec, and another 20 cycles of $94^0$C for 45 sec, $45^0$C for 45 sec, and $72^0$C for 60 sec. After the 40 cycles were complete the reactions were incubated at 72°C for 5 min then stored at 4°C or -20°C. Reactions were checked on a 3% agrose minigel (section 4.2.1.6.).

## 4. 2.10. PURIFICATION OF PCR PRODUCTS FOR SEQUENCING

PCR products (100 µl) (section 4.2.9.2.) were added to 100 µl of Wizard PCR Preps (Promega) Direct Purification Buffer, this mixture was vortexed briefly, 1 ml of Wizard PCR Preps DNA Purification Resin was added and the mixture vortexed briefly three times over a 1 min period. This mixture was then loaded onto a Wizard PCR Preps Minicolumn using a 3 ml syringe with a Leur tip, the Minicolumn washed with 2 ml of 80% isopropanol, centrifuged briefly to dry and the DNA was washed off the Minicolumn by centrifuging 50 µl of MilliQ water through the column at 15 000 g. The purified DNA was ready for subcloning and sequencing. The polyester system was also used for the purification of PCR products (section 4.2.7.2.).

### 4.2.11. DNA LIGATIONS

Before PCR products (94 bp DNA fragment amplified from pine genomic DNA) were ligated to plasmid M13mp18 circular DNA, both the PCR product and M13mp18 plasmid DNA together were digested with *Xba*I and *Eco* RI. The PCR product has restriction sites *Eco* RI and *Xba* I at its ends and can be inserted into the corresponding restriction sites *Eco* RI and *Xba* I in M13mp18 plasmid DNA (section 4.2.1.22.). As *Eco* RI required 2 times higher salt concentration than *Xba* I to achieve the best possible digestion, the PCR product and M13mp18 plasmid DNA together were digested first with *Xba* I for 90 minutes. Then an additional 50 mM NaCl and *Eco* RI were added to the same mixture to be digested for another 90 minutes. A 2-3 times molar excess of insert to vector was used in the digestion mixture. The digestion mixture was purified by ethanol precipitation method (section 4.2.3.2.). At least 20 ng of DNA insert (purified PCR product) and 20 ng of vector DNA were mixed with 2.0 µl of 10 x ligation buffer (New England Biolabs), 1.0 µl of undiluted T4-DNA ligase (New England Biolabs), and MilliQ water up to 20 µl. Ligation mixtures were left in a refrigerator overnight.

To check that ligation had occurred, 2.0 µl of the ligation mix was removed prior to addition of T4-DNA ligase, 2.0 µl of SDS loading buffer (section 4.2.1.6.) was added and the sample was examined on an agarose gel (section 4.2.6.) alongside a 2.0 µl sample (with 2.0 µl of SDS loading buffer) removed after addition of T4 DNA ligase and overnight ligation.

### 4.2.12. PREPARATION OF COMPETENT *E. COLI* CELLS

Five ml of LB broth and 5µl (Tetracycline, 50mg/ml) (section 4.2.1.1.) was inoculated with *E. coli* XL1 cells, and grown at 37°C with vigorous shaking overnight. 1 ml of overnight culture was added to 100 ml LB and grown at $37^0$C with shaking to mid-log phase ($OD_{600}$ 0.5-1.0, about 6 hours). 100 ml cultured cells were divided and transferred into two 50 ml falcon tubes. The falcon tubes were centrifuged at 6000 g for 10 minutes at $4^0$C. The supernatant was tipped out, and the precipitated pellet was suspended in 5 ml of cold ($4^0$C) 50 mM $CaCl_2$ and then 45 ml 50 mM $CaCl_2$ was added into each falcon tube. They were kept on ice for 30 minutes and centrifuged at 6 000 g

for 10 minutes at $4^0$C. The supernatant was tipped out, and the pellet was suspended in 2 ml 50 mM CaCl2 and stored at $4^0$C. The efficiency of cells with regard to transformation peaks at 24 hours. But it can be used right away or up to 2 days old.

### 4.2.13. TRANSFORMATION OF *E. COLI* WITH M13 BY HEAT SHOCK METHOD

1-10 µl DNA ligation mixture (section 4.2.11.) was added to 300 µl competent *E. coli* XL-1 cells in a 5 ml kimax tube on ice. The tube was kept on ice for 20-30 minutes, and then the tube was transferred onto a $42^0$C hot plate to heat-shock for 90 seconds. 50 µl 2% X-gal (in dimethylformamide), 20 µl 100 mM isopropyl thiogalactoside (IPTG) and 3.5 ml melted LB top agar (section 4.2.1.3.) were added into the tube and the mixture was immediately poured onto a LB agar plates (section 4.2.1.1.). The LB agar plates then were kept at $37^0$C over night.

### 4.2.14. DNA SUBCLONING

The process of subcloning typically involved recovery of DNA fragments from agarose gels (section 4.2.7.), digestion and ligation of DNA fragments with a suitable vector (section 4.2.11.) and the transformation of ligation mixtures into a suitable *E. coli* host using the heat shock method (section 4.2.13.). When M13mp18 was used as a vector for subcloning, blue/white selection (α-complementation) was employed to screen for putative recombinants (white plaques) in a *E. coli* XL-1 cell blue background. In this case, white plaques were picked up by toothpicks and put into 5 ml kimax tubes containing overnight cultured XL-1 cells and 2x YT media (section 4.2.1.2.). These kimax tubes were incubated with shaking at $37^0$C for 6 hours, and the cells were harvested by centrifugation for 5 minutes at 15, 000g. Supernatant containing single stranded template M13 DNA was transferred into new eppendorf tubes and was precipitated in PEG solution (30% PEG 8000, 1.6 M NaCl) for 15 minutes at room temperature. Single stranded DNA was then extracted with phenol/chloroform (section 4.2.3.1.), precipitated by 3 M Sodium Acetate and isopropanol/ethanol (section 4.2.3.2.).

The double stranded M13 DNA was also harvested from the cell pellet by centrifugation of the harvested XL-1 cells, suspension in STET buffer (section 4.2.1.5.) and isolation double stranded M13 DNA was done by the rapid boiling method (section

4.2.2.2. ). Recombinants were detected by gel electrophoresis (section 4.2.6.) of diagnostic restriction digests (section 4.2.5.) of double stranded plasmid DNA.

## 4. 2.15. DNA SEQUENCING

Sequenase Version 2.0 (USB), based on the dideoxy-mediated chain termination method of Sanger *et al* (1977), was used for DNA sequencing. 5.0 µl (0.2 µg/µl ) DNA template (single stranded M13 plasmid DNA with pine PCR products as inserts, section 4.2.11.) was mixed with 2.0 µl MilliQ water, 2.0 µl sequencing reaction buffer and 1.0 µl primer solution and primers were annealed to the template DNA at $65^0$C for 2 minutes and gradually cooled to < $35^0$C. While the annealing mixes were incubating the chain termination mixes were set up on a microtitre plate: one set of the four termination mixes per sequencing reaction; 2.5 µl of the appropriate d/ddNTP mix per well. After annealing, 1.0 µl of 0.1 M dithiothreitol, 2.0 µl of labelling mix diluted 5x in MilliQ water, 0.5 µl of [$\alpha$-$^{35}$S]dATP and 2.0 µl of Sequenase diluted 8x in TE (section 4.2.1.8.) were added to the annealing mix. These labelling reactions were incubated at room temp for 5 minutes, then 3.5 µl of the labelling reactions were transferred to each of the four termination wells of the microtitre dish prior to incubation at 37°C for 5 min. Sequenase stop solution (4.0 µl) was then added to each well and the reactions stored at -20°C.

## 4. 2.16. POLYACRYLAMIDE GEL ELECTROPHORESIS OF SEQUENCING REACTIONS

Sequencing reactions (section 4.2.15.) were separated by polyacrylamide gel electrophoresis (PAGE). Sequencing gels were poured with 60 ml of acrylamide mix (section 4.2.1.12) containing 36 µl of TEMED (NNN'N'-Tetramethylethylenediamine) (BDH) and 360 µl of 10% (w/v) ammonium persulphate. Once gels had set they were pre-run for 15-60 min with constant power (65 W) in 1 x TBE sequencing buffer (section 4.2.1.14.). Sequencing reaction mixtures were then denatured at 75°C for 2 min and 3 µl of the sequencing reaction mixtures were loaded onto the sequencing gel. These reactions were run until the first dye front from these reactions (the bromophenol blue) had run off the gel (short runs, typically 2 h). The gel was then disassembled,

fixed in a solution containing 10% acetic acid, 10% ethanol for 30 min, dried for 35 min under vacuum at 80°C then autoradiographed overnight.

## 4.2.17. DNA SEQUENCE ANALYSIS

Fourteen DNA sequences amplified by PCR from *Pinus radiata* (section 4.2.9.2.) were determined by methods described in section 4.2.16. They were aligned with the conserved *AGAMOUS* MADS box region using a PILEUP programme of GCG (Genetic Computer Group, Inc. version 7, April 1991) with a gap weight of 5.0 and a gap length weight of 0.30.

Seven representative DNA sequences (Pm1-4, Pm6, Pm7, and Pm13) (Pm: pine mads-box) isolated by PCR from *Pinus radiata* were also aligned with the corresponding conserved DNA sequences of other MADS-box genes, such as *AGL-1* (GenBank M55550), *AGL-2* (GenBank M55551), *AGL-4* (GenBank M55552), *AGL-5* (GenBank M55553) and *AGL-6* (GenBank M55554) (Ma *et al.*, 1991), *Ap1*(GenBank Z16421) (Mandel *et al.*, 1992), *AP3* (GenBank M86357) (Jack *et al.*, 1992), *AGAMOUS* (*AG*) (GenBank X53579) (Yanofsky *et al.*, 1990) of *Arabidopsis thaliana*, *BAG1* of *Brassica napus* (GenBank M99415) (Mandel, *et al.*, 1992 b), *DMU* of *Drosophilia melanogaster* (GenBank UO3292), *FBP*2 of *Petunia hybrida* (GenBank M91666) (Angenent *et al.*, 1992), *OM*1 of orchid (*Aranda deborah*) (EMBL X69107) (Lu *et al.*, 1993), *OsMADS*1of rice (*Oryza sativa*) (GenBank L34271) (Chung *et al.*, 1994), *DAL*1 (EMBL X80902), *DAL2* (EMBL X79280) and *DAL3* (EMBL 79281) of Norway spruce (*Picea abies*) (Tandre *et al.*, 1995), *TAG*1 of tomato (*Lycopersicon esculentum*) (Pnueli *et al.*, 1994) and *ZAG*1 (GenBank L18924), *ZAG2* (GenBank L18925) of corn (*Zea mays*) (Schmidt *et al.*, 1993). A consensus sequence was calculated by the PRETTY programme of GCG. Sequences (except those of *DAL*1, 2, 3, *OM1* and *TAG1* ) were obtained from GenBank sequence database (release 72.0 ) or by a search of GenBank (release 82.0) at the National Centre for Biotechnology Information with NCSA Mosaic.

A dendrogram tree showing the relatedness of DNA sequences from *Pinus radiata* and those MADS box genes from various species mentioned above was also made using the PILEUP programme of GCG with a gap weight of 5.0 and a gap length weight of 0.3.

The deduced amino acid sequences of seven DNA sequences (Pm 1-4, Pm 6, Pm 7, and Pm 13) isolated from *Pinus radiata* were determined using the TRANSLATE programme of GCG, and the translational reading frame was decided according to the sequence similarity between *AGAMOUS* MADS-box region and pine DNA sequences amplified by PCR from this study. These deduced amino acid sequences were also aligned with

deduced amino acid sequences of MADS box genes, *AG*, *AGL*1-6, *AP1*, *AP*3, *DAL*1-3, *FBP*2, *OM1*, and *OsMADS*1.

# 4.3 RESULTS

## 4. 3.1. ISOLATION AND QUANTIFICATION OF THE GENOMIC DNA FROM NEEDLE FASCICLE TISSUE OF *Pinus radiata*

Genomic DNA was isolated using a CTAB miniprep method described in section 4.2.2.1. The isolated genomic DNA from pine tissues was treated with 100 μg/ml RNase (section 4.2.1.13) first, and then the spectrophotometric method (section 4.2.4.1.) was used to estimate the DNA purity. The result is shown in Table **4.2**.

Table **4.2** Spectrophotometric estimation of the purity of DNA solution extracted from pine tissues.

| DNA samples | Pine DNA sample tube 1 | Pine DNA sample tube 2 | Pine DNA sample tube 3 | Mean value of three samples | Pure DNA $OD_{260}/_{280}$ value |
|---|---|---|---|---|---|
| $OD_{260}/OD_{280}$ value | 1.77 | 1.88 | 1.96 | 1.87 | 1.80 |

The average $OD_{260}/OD_{280}$ value from three tubes is 1.87. This data was quite close to the pure DNA $OD_{260}/OD_{280}$ value of 1.8, indicating the isolated pine genomic DNA was relatively pure.

The DNA concentration was quantified using the fluorometric method and by comparison with the fluorescence of lambda DNA concentration standards on an ethidium bromide-stained agrose gel (section 4.2.4.2, 3). Combining the results of these two methods, the average DNA concentration of sample DNA solution of three tubes was about 50ng/μl. The total DNA solution volume was 1.5 ml, so close to 75 μg genomic DNA was obtained. The average yield of the genomic DNA from the fresh needle fascicle tissue was 50ng/mg .

## 4. 3.2. RESTRICTION ENDONUCLEASE DIGESTION OF PINE GENOMIC DNA

The quality of the isolated genomic DNA from the pine needle fascicle tissue was evaluated by digestion with restriction endonucleases *Eco* RI and *Bam* HI (section 4.2.5.). The original batch of pine genomic DNA was only partially digested, whilst after an extra round of precipitation of DNA with ethanol (section 4.2.3.2.), digestion yielded an even smear of DNA fragments after restriction, indicating that DNA was digested completely (Fig 4.1).

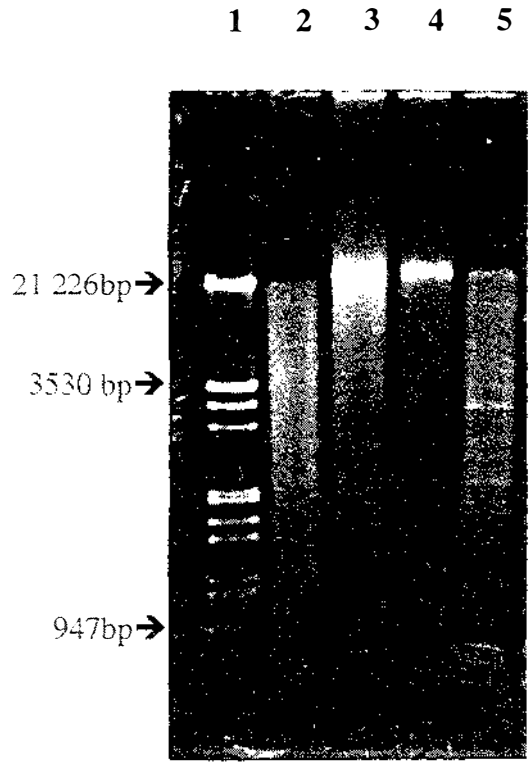



Fig 4.1. Restriction endonuclease digestion of 125 ng pine genomic DNA and 125 ng *Arabidopsis* genomic DNA. Three samples of pine genomic DNA (A,B,C) from the same extraction are shown. One was treated with an extra round of ethanol precipitation.

**1** : Standard λ DNA digested with *Eco* RI and *Hind* III (for standard marker sizes see section 3.2.1.19.)

**2** : Pine genomic DNA treated with an extra round of ethanol precipitation, digested with *Eco* RI, tube A.

**3** : Pine genomic DNA from sample tube B before an extra round of ethanol precipitation, digested with *Eco* RI.

**4** : Pine genomic DNA from sample tube C before an extra round of ethanol precipitation, digested with *Bam* HI.

**5**: *Arabidopsis* genomic DNA without being treated with extra ethanol precipitation, digested with *Eco* RI.

Pine genomic DNA treated with an extra round of ethanol precipitation was used for the Southern blot study and as a template for PCR amplification. The digested *Arabidopsis* genomic DNA was not treated with an extra round of ethanol precipitation, but showed a complete and clear digestion.

### 4. 3. 3. PROBE PREPARATION FOR SOUTHERN HYBRIDISATION STUDY

A 968 bp *AG* cDNA fragment from *Arabidopsis* (*Eco* RI fragment from the cDNA clone pGEM7Z(+)) (section 4.2.1.17.) and a 1451 bp *LFY* cDNA fragment from *Arabidopsis* (*Bam* HI + *Kpn* I fragment from the cDNA clone pBluescript KS+($Amp^r$ )) (section 4.2.1.18.) were used as probes for hybridisation with pine genomic DNA. *AG* and *LFY* plasmid DNA were extracted using a rapid boil method (section 4.2.2.2.), the *AG* and *LFY* cDNA fragments were cut out of the vector DNA by digestion with *Eco* RI or *Bam* HI + *Kpn* I respectively (section 4.2.5.) (Fig 4.2.). *AG* and *LFY* cDNA fragments were recovered from agarose gels (section 4.2.7.) and were then purified through a Glassmax™ DNA isolation spin cartridge system (section 4.2.7.1.). About 50 ng of *AG* and *LFY* cDNA fragments were obtained with this method. These two cDNA fragments were then radioactively labelled (section 4.2.8.2.) (section 4.2.8.3.) and hybridised with pine genomic DNA (section 4.2.8.4.).

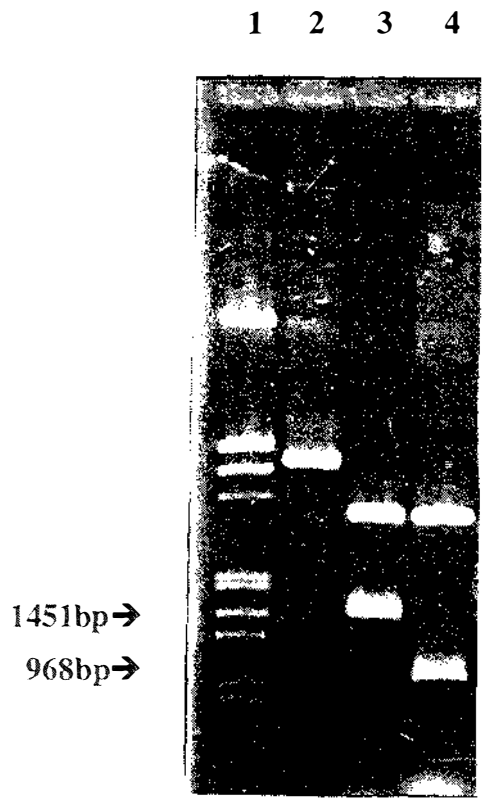



Fig 4.2. Isolating the *Arabidopsis AGAMOUS* cDNA insert from pGEM7Z(+) plasmid DNA and *LEAFY* (*LFY*) cDNA from pBluescript by restriction endonuclease digestion.

**1**: Standard λ DNA digested with *Eco* RI and *Hind* III.

**2**: pBluescript plasmid DNA with *LFY* cDNA insert digested with *Kpn* I.

**3**: pBluescript plasmid DNA with *LFY* cDNA insert digested with *Bam* HI and *Kpn* I, showing the 1451 bp long *LFY* cDNA insert (arrow).

**4**: pGEM7Z(+) plasmid DNA with *AG* cDNA insert digested with *Eco* RI, showing the 968 bp long *AGAMOUS* cDNA insert (arrow).

## 4. 3. 4. DETERMINING THE QUANTITY OF THE PINE GENOMIC DNA REQUIRED FOR THE SOUTHERN BLOTTING AND HYBRIDISATION.

As *AGAMOUS* and *LEAFY*-like genes that control floral development are expected to be in low- or single-copy number and the *Pinus radiata* genome is seven times larger than the human genome (Neale and Williams, 1991) and a hundred times larger than that of *Arabidopsis* (Carlson *et al.*, 1991), a substantial amount of DNA was required for this study.

0.35 µg *Arabidopsis* genomic DNA digested with *Eco* RI was loaded as positive control, and 35 µg pine genomic DNA digested with *Eco* RI and *Bam* HI was loaded per lane. 0.5 ng and 0.05 ng of digested *AG* and *LFY* cDNA sequences were also used as further positive controls. The digested 35 µg pine genomic DNA, *Arabidopsis* genomic DNA, and diluted *AG* and *LFY* plasmid DNA were separated by overnight gel electrophoresis (Section 4.2.6.) and transferred to the Nylon membrane by Southern (capillary) blotting (Section 4.2.8.1.). Fig 4.3 shows the digested DNA after electrophoresis and before capillary blotting. Because only small amount of DNA was loaded, no DNA bands were detected in Lanes 6, 7, and 9. One band with the size of *AG* plasmid vector DNA was seen in lane 9, this was probably due to the fact that the DNA was not suspended effectively which resulted in incorrect determination of DNA concentration and consequently, more than 0.5 ng DNA was loaded in lane 9 on the gel.

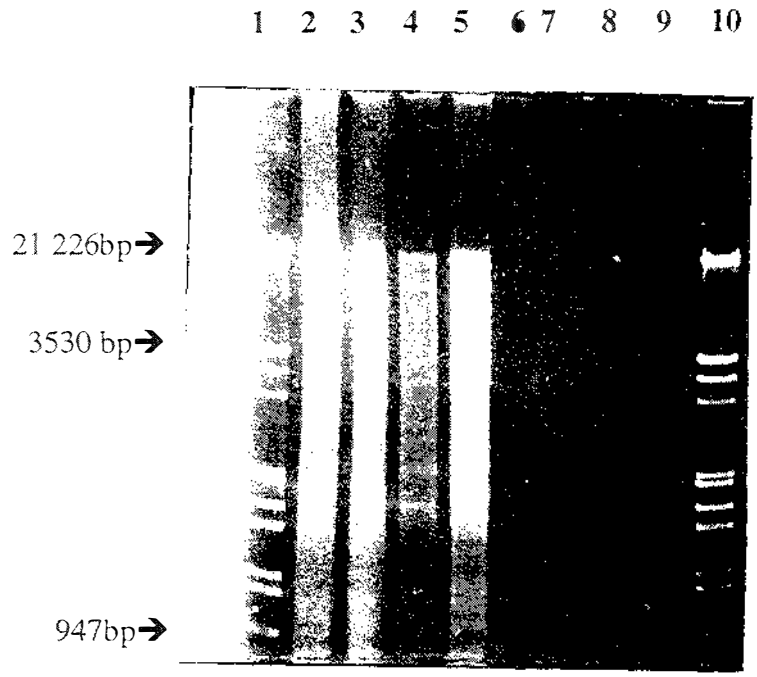



Fig 4.3. Overnight gel electrophoresis using a 1% agarose TAE (section 3.2.1.6.) gel.

**1,10** : 1 μg λ DNA digested with *Eco* RI and *Hind* III.

**2** : 35 μg pine genomic DNA digested with *Eco* RI.

**3** : 35 μg pine genomic DNA digested with *Bam* HI.

**4,5** : 0.35 μg (lane 4) and 1.2 μg (lane 5) *Arabidopsis* genomic DNA digested with *Eco* RI.

**6,7** : 0.5 ng (lane 6) and 0.05 ng (lane 7) *LFY* plasmid DNA digested with *Bam* HI+*Kpn* I.

**8,9** : 0.5 ng (lane 8) and 0.05 ng (lane 9) *AG* plasmid DNA digested with *Eco* RI.

## 4. 3. 5. DETERMINING CONDITIONS FOR SOUTHERN HYBRIDISATION

Southern blots ( section 4.3.4.) were hybridised to [$\alpha$-$^{32}$P] dCTP-labelled *AG* and *LFY* cDNA fragments in hybridisation buffer (section 4.2.1.9.) containing 3 x SSC at $55^0$C, $60^0$C and $65^0$C then washed at the same temperatures in 3 x SSC, 0.1% SDS (three washes of 30 minutes each ) and autoradiographed for seven days. Hybridisations and washes at $60^0$C gave the clearest signal with minimal background. The result is shown in Fig 4.4.

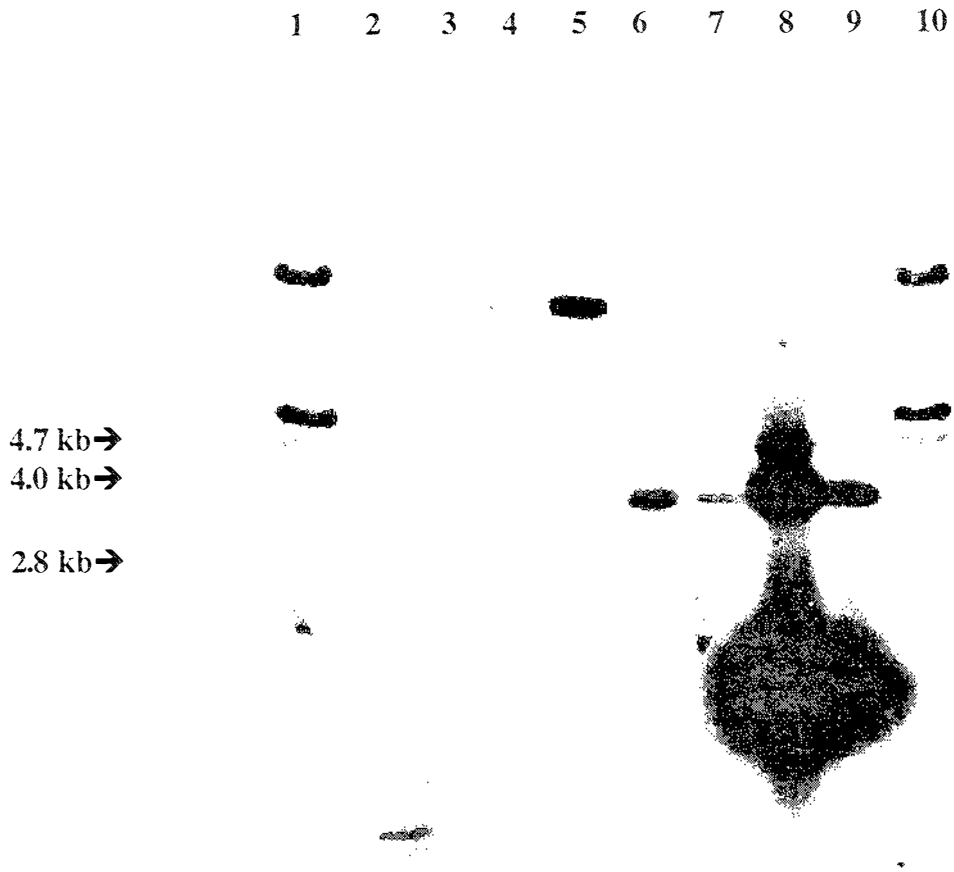



Fig 4.4. An autoradiograph showing the result of the Southern hybridisation between *Arabidopsis AGAMOUS* cDNA probe and digested pine genomic DNA (arrows ). This blot was made from the gel which is shown in Fig 4.3.

**1,10** : 1 μg λ DNA digested with *Eco* RI and *Hind* III.

**2** : 35 μg pine genomic DNA digested with *Eco* RI.

**3** : 35 μg pine genomic DNA digested with *Bam* HI.

**4,5** : 0.35 μg (lane 4) and 1.2 μg (lane 5) *Arabidopsis* genomic DNA digested with *Eco* RI (positive control).

**6,7** : 0.5 ng (lane 6) and 0.05 ng (lane 7) *Arabidopsis LFY* plasmid DNA digested with *Bam* HI+*Kpn* I.

**8,9** : 0.5 ng (lane 8) and 0.05 ng (lane 9) *Arabidopsis AG* plasmid DNA digested with *Eco* R I (positive control).

Fig 4.4 shows two distinct bands of 4.7 kb, 2.8 kb and one less distinct band of 4.0 kb in lane 2, and two distinct bands of 4.7 bp, 4.0 kb and one less distinct band of 2.8 kb in lane 3. The causes for this ambiguous result were not clear. So this result can only provide a tentative evidence of hybridisations between *AG* cDNA sequence and digested pine genomic DNA. Some non-specific bands were also visible in the background, but they all disappeared when the blot was washed under a high stringency condition (1 x SSC wash at $65^0$C). After stripping hybridised DNA off Southern blots, it was hybridised to [$\alpha$-$^{32}$P]dCTP-labelled *LFY* cDNA fragment at $60^0$C and the hybridised blot was washed at $60^0$C. No hybridised signals were detected.

As the Southern blot study can not provide convincing evidences of the existence of *AG*-like genes in the pine genome, PCR technique was subsequently applied to isolate *AG*-like DNA sequences from the pine genome.

## 4. 3. 6. AMPLIFICATION OF PINE GENOMIC DNA BY THE POLYMERASE CHAIN REACTION (PCR)

A set of degenerate primers were designed targeting the conserved MADS box region of *AG*-like genes in the *Pinus* genome (section 4.2.9.1 and 4.2.9.2.). Due to the degenerate nature of primers, a low stringency annealing temperature was chosen for this PCR study: $37^0$C for 20 cycles and $45^0$C for 20 cycles (section 4.2.9.1.). This PCR condition was modified from those used in D. White's Laboratory at AgResearch Institute, Palmerston North, New Zealand . Primers designed for this study targeted the conserved region of the MADS box domain between amino acids RQVT and VLCDAE (section 4.2.9.1.). The amplified fragments were expected to be about 94 bp (section 4.2.9.2.). Fig 4.5 shows the result of this study. The PCR product bands revealed on 3% agarose gel were quite weak (lane 3, Fig 4.5.) despite the low annealing temperature condition. But an alteration of primer and template DNA concentration had a significant impact on the result. Fig 5 also shows that an increase in both template DNA and primer concentration produced much clearer bands (lanes 4 and 5, Fig 4.5). Excess primers were also shown in the lower end of each lane, including the negative control lane in Fig 4.5.

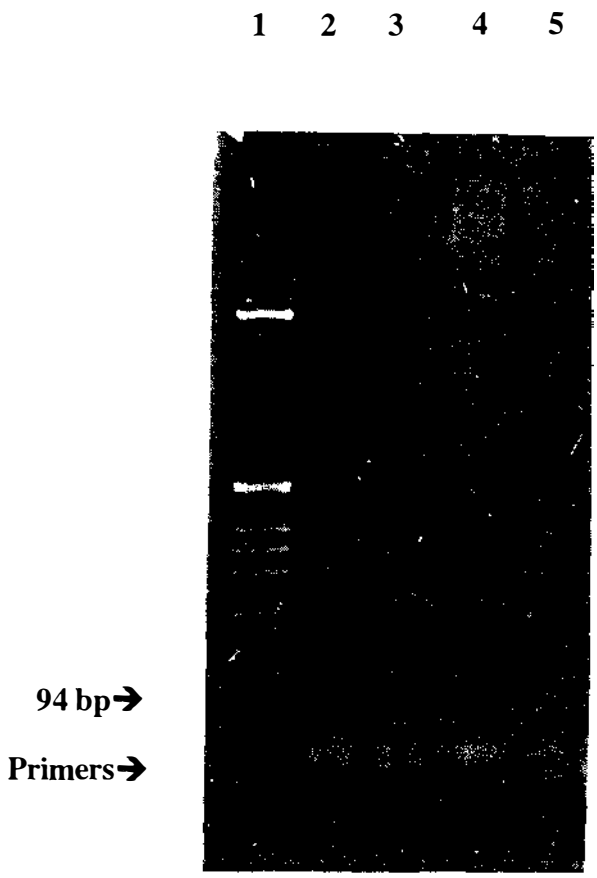



Fig 4.5. 94 bp PCR fragments (arrow) amplified from pine genomic DNA, using degenerate primers targeting the conserved site of the MADS box region were revealed on 3% agarose gel.

Lanes **3-5** : 3 µl of a 100 µl PCR reaction mixture with different amounts of template DNA and primers was loaded on each lane.

**1** : Standard pBR 322 plasmid DNA digested with *Hinf*I.

**2** : Negative control with no template DNA added to 100 µl PCR reaction mixture.

**3** : 200 ng template pine DNA and 4 µl (20 µM) primers were added to 100 µl PCR reaction mixture.

**4** : 800 ng template pine DNA and 8 µl (20 µM) primers were added to 100 µl PCR reaction mixture.

**5**: 400 ng template pine DNA and 8 µl (20 µM) primers were added to 100 µl PCR reaction mixture.

In order to isolate enough 94 bp long *AG* related DNA sequences from *Pinus radiata* for subcloning and sequencing analysis, PCR products from the first 40 cycles of amplification, shown in lanes 3 and 4 in Fig 4.5 were used as template DNA and amplified again for another 40 cycles. The resolved bands were much more distinct and easier to isolate (arrow Fig 4.6.). Fig 4.6 also showed some different bands in addition to 94 bp, these probably were the resulting bands of the binding between degenerate primers and the template DNA, which are not likely to be related to MADS-box genes in the *Pinus* genome.

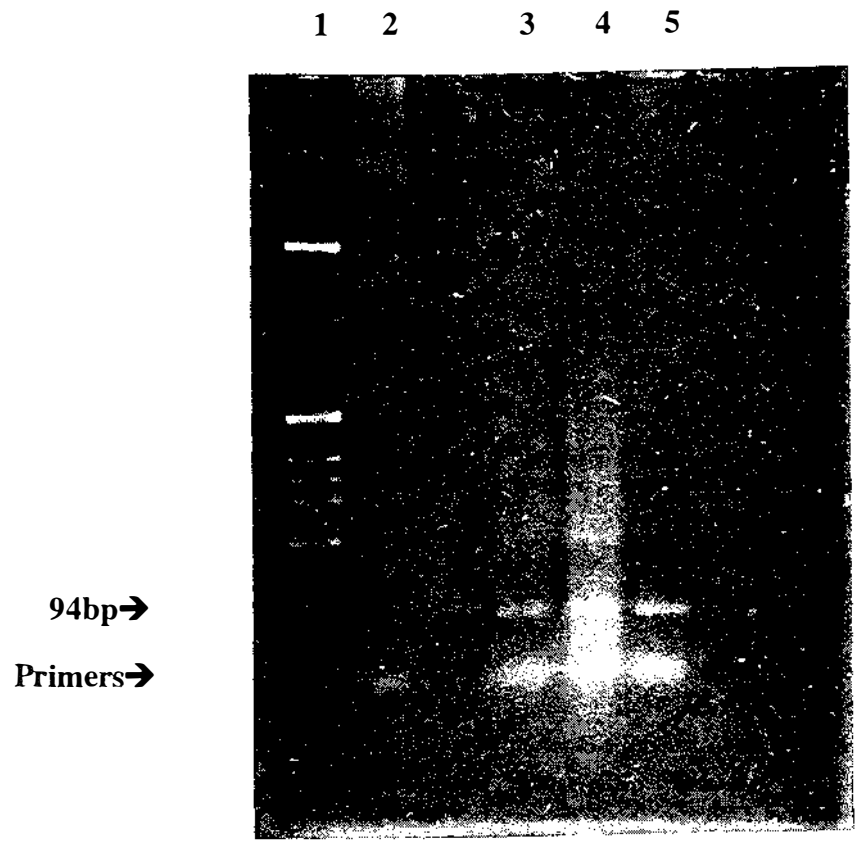



Fig 4.6. PCR products shown in Fig 4.5 were used as template DNA and amplified again for another 40 cycles. Much more distinct bands of 94 bp (arrow) were visible on a 3% agarose gel. Lanes **3-5** : 3 µl of a 100 µl PCR reaction mixture with 8 µl (20 µm) primers and template DNA from different sources was loaded on each lane.

**1** : Standard pBR 322 plasmid DNA digested with *Hinf* I.

**2** : Negative control with no template DNA added in the 100 µl PCR reaction mixture.

**3** : 10 µl PCR product mixture from the first round shown on lane 3 in Fig 4.5. were used as template DNA in the 100 µl PCR reaction mixture.

**4** : 10 µl PCR product mixture from the first round shown on lane 4 in Fig 4.5. were used as template DNA in the 100 µl PCR reaction mixture.

**5** : 250 ng *Arabidopsis* genomic DNA was used as template DNA (positive control) in the 100µl PCR reaction mixture.

The PCR product showed in lanes 3 and 4 in Fig 4.6 was treated with 0.3 M sodium acetate and precipitated with 95% ethanol (section 4.2.3.2.). Then precipitated DNA pellets were resuspended in 10 µl TE buffer at $55^0$C (section 4.2.1.7.) and loaded onto a 3% SeaPlaque agarose (low melting point) gel and separated by gel electrophoresis at $4^0$C (section 4.2.6.). To avoid the contamination from amplified bands of different sizes, the targeted band of 94 bp (arrow , Fig 4.7) was excised under UV illuminator. pBR322 plasmid DNA digested with *Hinf* I was used as a ladder (section 4.2.20.). The excised bands were positioned in a polyester filter (Struhl 1994) and 94 bp DNA fragments were recovered through a centrifugation at 15 000 g for 5 minutes (section 4.2.7.2.) and purified by ethanol precipitation (section 4.2.3.2.). Subsequently Wizard PCR Preps were also used for further purifying the PCR products (section 4.2.10.). The purified PCR products were tested on a 3% agarose gel. About 50 ng purified PCR products (amplified pine DNA sequences related to *Arabidopsis* MADS-box) were obtained and they were used for subcloning and sequencing analysis.

PCR is a highly sensitive method, if any contaminating target DNA appeared in the PCR reaction mixture, it is possible that it will be amplified, and the result would therefore not be reliable. Contamination is more likely to happen when a DNA sample which is homologous to the primer is used as a positive control. In this case, extreme care has to be taken to avoid cross-contamination.

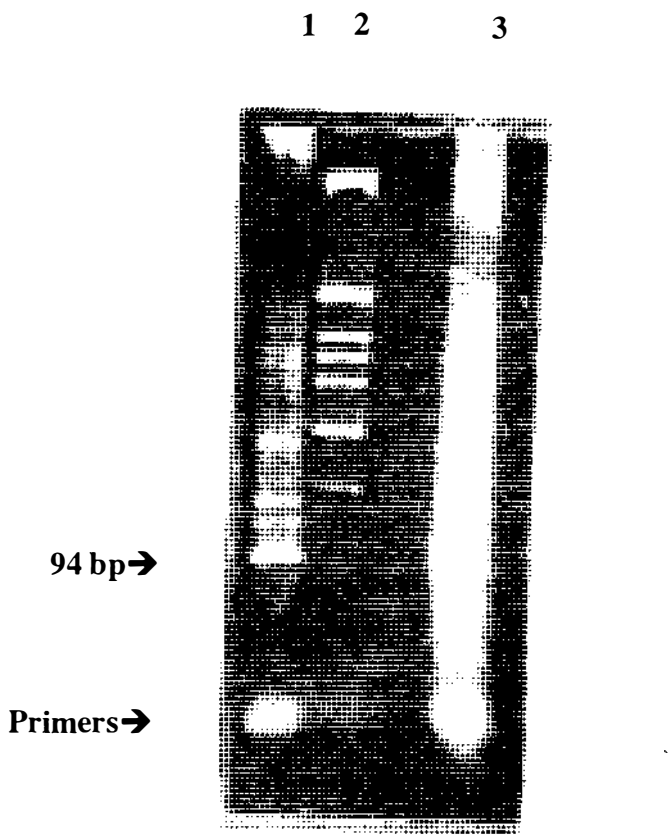



Fig 4.7. PCR products shown on lanes 3 and 4 in Fig 4.6 were concentrated 10 fold and separated on a 3% SeaPlague agarose (low melting point) gel at $4^0$C. The non-specific bands are also seen clearly in this figure, but only the targeted 94 bp band (arrow) was excised and purified.

**1** : 10 µl concentrated PCR product mixture made from the PCR reaction shown on lane 3 in Fig 4.6 was loaded.

**2** : Standard pBR 322 plasmid DNA digested with *Hinf* I.

**3** : 10 µl concentrated PCR product mixture made from the PCR reaction shown on lane 4 in Fig 4.6 was loaded.

## 4. 3.7. DNA SUBCLONING

Purified PCR products (95 bp amplified pine DNA fragments) and M13mp18 plasmid DNA were digested with *Eco* RI and *Xba* I and ligated with T4-DNA ligase (section 4.2.11). Prepared competent *E. coli* XL-1 cells (section 4.2.12.) were transformed with ligated M13mp18 containing pine DNA inserts using the heat shock method (section 4. 2.13.). Putative recombinants (white plaques) were selected and grown in LB media (section 4. 2.1.1) and 2 xYT media (section 4.2.1.15) over night. Single stranded M13mp18 plasmid containing pine DNA inserts were first precipitated in PEG solution and then extracted using the phenol/chloroform method (section 4. 2.14.). Single stranded recombinants growing in LB media and YT media were tested by gel electrophoresis (section 4.2.6.). Putative recombinants [M13mp18 plasmid DNA containing pine DNA inserts (PCR products)] were selected and grown in LB media and 2xYT media respectively. Single stranded recombinants grown in LB media and YT media were tested on a 1% agarose gel. It was not possible to distinguish recombinants from the wild type (lane **8** on the right hand gel), because of the short length of the insert. The result showed that phage grown in YT media gave a much better yield of ssDNA than those grown in LB media (Fig. 4.8). The reason for the appearances of some extra smaller bands is unknown.

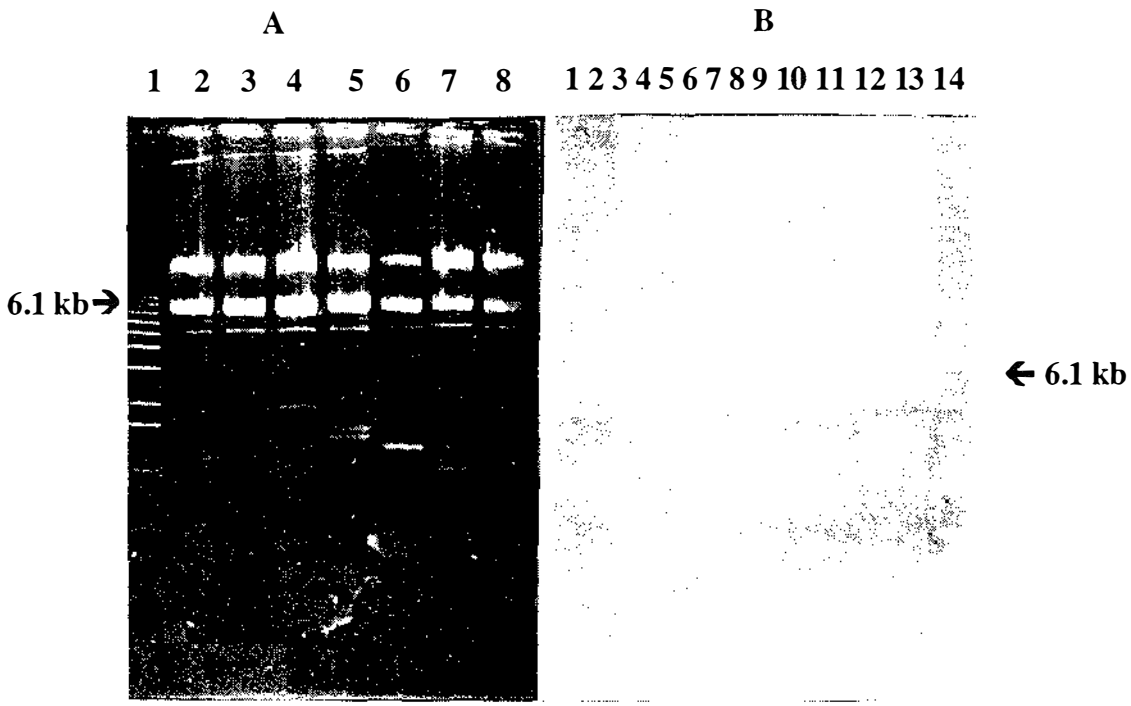



Fig 4.8. Gel **A** showing the single stranded putative recombinants (arrow) grown in LB media.

**1** : 1 kb ladder.

**2-8** : 3 µl DNA from 7 single stranded putative recombinants.

Gel **B** showing the single stranded putative recombinants (arrow) grown in 2 x YTmedia.

**1, 14** : 1 kb ladder.

**2-7** : 3 µl DNA from 6 single stranded putative recombinants.

**8** : 3 µl wild type M13mp18 plasmid DNA with no foreign DNA insert.

**9-13** : 3 µl DNA from 5 single stranded putative recombinants.

## 4. 3. 8. DNA SEQUENCING AND SEQUENCE ANALYSIS

Single stranded recombinant M13 DNA was sequenced using a Sequenase Version 2.0 protocol based on the dideoxy-mediated chain termination method of Sanger (1977) (section 4.2.15). Nucleotide sequences were revealed by PAGE Gel Electrophoresis (section 4.2.16.). Fourteen sequences were identified as true recombinants with both restriction sites (*Eco* RI and *Xba* I) at both ends. Five sequences were self-circled M13mp18 single stranded DNA with no insert. One sequence had correct restriction sites at both ends, but the sequence between two restriction sites was nine base pairs longer than those fourteen true recombinants and its sequence was quite different from other MADS-box genes . It was probably a product of the binding between degenerate primers with pine DNA sequences unrelated to MADS-box genes during PCR amplification. These 14 DNA sequences isolated from *Pinus radiata* were aligned with the conserved *AGAMOUS* MADS box region from *Arabidopsis* by PILEUP of the GCG package with a gap weight 5.0 and a gap length weight of 0.30 and shown in Fig 4.9.

The alignment of these sequences with the conserved *AGAMOUS* MADS box region showed that they all shared a different degree of identity to the conserved MADS-box region of the *AGAMOUS* gene. Where different nucleotides in these pine DNA sequences occurred, they usually occurred at the third codon (Fig. 4.9). Primer regions were not representative of the accurate sequence data from pine DNA, because the primers used in this study are degenerate. DNA sequences were divided into seven different groups based on their sequences excluding the primer region. These seven sequences were represented by Pm1, Pm2, Pm3, Pm4, Pm6, Pm7 and Pm13, and they were aligned with eighteen MADS-box DNA sequences shown in Fig 4.10.

The relatedness of DNA sequences (Pm1-4, 6 and Pm13 ) with eighteen other MADS-box DNA sequences was analysed, using a PILEUP programme of GCG. This analysis showed clustering relationships used to determine the order of pairwise alignments and created the final alignment shown in Fig 4.11.


Fig 4.9. Fourteen (Pm1-Pm14) DNA sequences isolated from the Genomic DNA of *Pinus radiata* by PCR, using primers based on the conserved MADS-box region were aligned with the conserved MADS-box DNA sequences of *AG* (Yanofsky *et al.*, 1990) (*Arabidopsis thaliana*) by PILEUP of the GCG package with a gap weight of 5.0 and a gap length weight of 0.3. **Blue** highlighted regions are the primer regions, nucleotides different from *AGAMOUS* sequence are highlighted **cyan** in primer regions. Nucleotides identical to *AGAMOUS* sequence between two primers are highlighted **green**, nucleotides different from *AGAMOUS* are highlighted **red**.

```
AG          .G...A..T. .A........ ...C...... .....C.... .....
BAG1        .G...A.... .A........ ...C...... .....C..A. .....
AGL-5       .G...A..AC .C........ A..C...... .......... .....
AGL-1       .G...A..AC .C......C. T..C...... ........A. .....
AGL-4       GCT..A..T. .A........ ......A... .......... .T...
Pm4         .G........ .......A.. A......... .....C.... ....G
Pm13        .G........ .......A.. A......... .......... ....G
Pm3         .C........ .......G.. A......... .......... .....
AGL-6       .CA..AA.A. .A..C..... .......... .......... .T...
Pm2         .CT....... .......G.. A......... .......... .....
DAL3        .CT....... ...C..GC.. .......... ....C..... ....G
AGL-2       GCA.....T. ....C..... .T........ .........AT .G...
ZAG1        .G.......C .C..C..CC. C..C.....G ..G..C.... ....C
ZAG2        .G.......C .C.....GC. C..C.....G ..G..C.... ....C
OsMADS1     GC........ ....C..CC. ...C.....G ..C..C.... ....C
DAL1        .CG....... ....C..AC. .........G ..G..C.... ....G
TAG1        .G....A.GC .C........ ...T..A..G .........AT .G...
AP1         ACG..AA.A. .AGC....C. TT........ ...C.....A .....
AP3         .CA...A.A. .......... AT.C...... ..AC...... ..A.G
DAL2        .GT......C .A........ AT.......G ..G.....AT .A..A
OM1         GC.......C ....AC.CC. T..C.....G ..C....... ....C
FBP2        GCT...A.A. .A.....AC. AT....A... ........A. .T...
Pm6         .CG.....GC ...TG..G.. ...T..A..G ..AC.G.... .T.TC
Pm1         .CG.....GC ...TG..G.. ...T..A..G ..AC.G.... .T..C
DMU         AA........ A.TTC..CG. .A.......G ..C..C.... .G..C
Pm7         TTTC.TT.T. A.G.ATT.CA T..ATGTG.T TG.C.CTCTA .GC..
CONSENSUS   T-CAAGCGCA GGAATGGTTT GCTGAAGAAA GCTTATGAGC TCTCT
```

Fig 4.10. Sequence comparison of seven (**Pm1, Pm2, Pm3, Pm4, Pm6, Pm7, and Pm13**) DNA sequences isolated from the Genomic DNA of *Pinus radiata* by PCR with other MADS-box DNA sequences. Primer regions of these seven DNA sequences from *Pinus radiata* were excluded. These MADS-box DNA sequences are from *AG* (Yanofsky *et al.*, 1990), *AG L*1-6 (Ma *et al* ., 1991), *AP*1 (Mandel *et al*., 1992), *AP*3 ( Jack et al., 1992) (*Arabidopsis thaliana*), *OM*1 (Lu *et al*., 1993) (*Aranda deborah*), *BAG*1 (Mandel *et al*., 1992 b) (*Brassica napus*), *DMU* (GenBank, UO 3292) (*Drosophilia melanogaster*), *FBP*2 (Angenent *et al*., 1992) (*Petunia hybrida*), *DAL*1, *DAL*2 and *DAL*3 (Tandre *et al*., 1994) (*Picea abies*), *OsMADS*1 (Chung *et al*., 1994) (*Oryza sativa*), *TAG*1 (Pnueli et al., 1994) (*Lycopersicon esculentum*), *ZAG*1 and *ZAG*2 (Schmidt *et al*., 1993) (*Zea mays*). A consensus sequence highlighted **green** was calculated by PRETTY of the GCG package and plotted below the sequences, deviations from that consensus are highlighted **red**.

A table was made to show the percentage identity between seven pine DNA sequences amplified by PCR excluding primer regions and eight *Arabidopsis* MADS-box DNA sequences, using the BESTFIT program of the GCG. *Arabidopsis* MADS-box DNA sequences used in this comparison have a same length as pine DNA sequences.

**Table 4.3. The percentage identity between seven pine DNA sequences (Pm1-4, Pm6-7 and Pm13) and eight *Arabidopsis* MADS-box DNA sequences (*AG*, *AGL*-1, 2, 4, 5, 6, *API* and *AP3*).**

| % identity | Pm1 | Pm6 | Pm2 | Pm3 | Pm4 | Pm13 | Pm7 |
|---|---|---|---|---|---|---|---|
| *AG* | 62.2 | 60 | 80 | 82.2 | 84.4 | 82.2 | 35.6 |
| *AGL-1* | 62.2 | 60 | 75.5 | 77.8 | 75.5 | 77.8 | 35.6 |
| *AGL-2* | 60 | 57.8 | 77.8 | 77.8 | 71.1 | 73.3 | 35.6 |
| *AGL-4* | 66.7 | 64.4 | 82.2 | 80 | 73.3 | 75.5 | 33.3 |
| *AGL-5* | 62.2 | 60 | 82.2 | 84.4 | 82.2 | 84.4 | 28.9 |
| *AGL-6* | 62.2 | 60 | 80 | 80 | 75.5 | 75.5 | 31.1 |
| *AP3* | 64.4 | 62.2 | 77.8 | 77.8 | 75.5 | 77.8 | 28.9 |
| *API* | 55.6 | 51.1 | 68.9 | 68.9 | 64.4 | 64.4 | 35.6 |

The percent sequence identity was calculated using the BESTFIT program from the GCG sequence analysis software package. *AG* (Yanofsky *et al.*, 1990), *AGL*-1-6 (Ma *et al*, 1991), *ApI* (Mandel *et al* ., 1992) and *AP3* (Jack *et al.*, 1992).

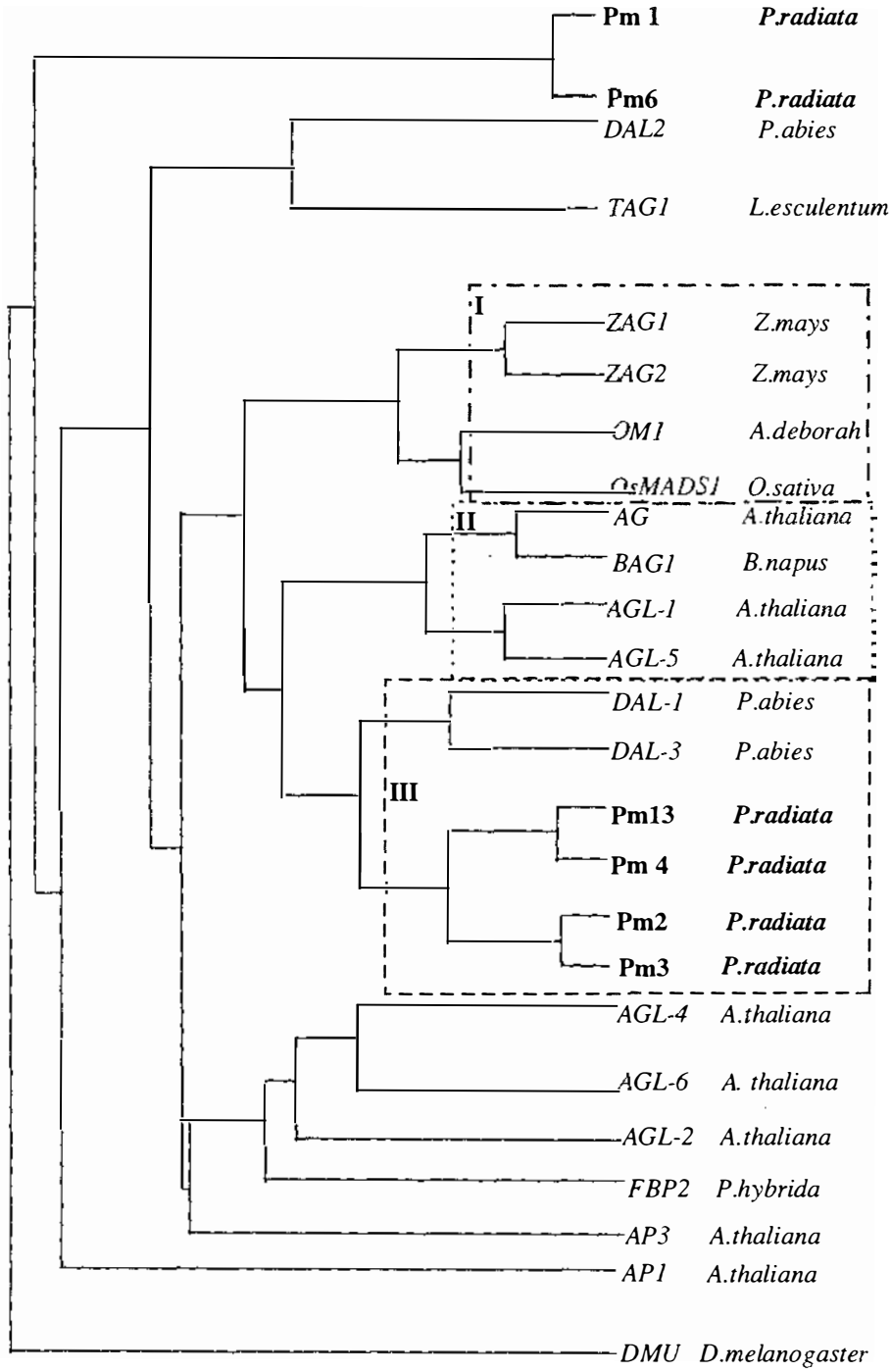



Fig 4.11. A dendrogram based on the pairwise sequence alignment, showing the relatedness of six pine DNA sequences (**Pm1, Pm2, Pm3, Pm4, Pm6, and Pm13** ) with MADS-box DNA sequences from other species. This dendogram used a PILEUP program of the GCG package, distance along the horizontal axis is proportional to the difference between sequences. Box **I** include four genes from monocotyledon species, box **II** include *AG* gene and three genes closely related to *AG* from dicotyledon species, and box **III** include four pine DNA sequences from this study and two genes from two conifer species.

## 4. 3. 9. ANALYSIS OF THE DEDUCED AMINO ACID SEQUENCE OF THE CONSERVED MADS-BOX FROM *Pinus radiata*

The deduced amino acid sequences of seven DNA sequences (Pm 1-4, Pm 6, Pm 7, and Pm 13) amplified from *Pinus radiata* were determined using the TRANSLATE programme of GCG. They were also aligned with deduced amino acid sequences of MADS box genes, *AG*, *AGL*1-6, and *AP*3 of *Arabidopsis thaliana*, *OM1* of *Aranda deborah*, *FBP*2 of *Petunia hybrida*, *DAL1*, *DAL2* and *DAL3* of *Picea abies*, and *OsMADS1* of *Oryza sativa* (Fig 4.12).

| | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Pm7** | R | Q | V | T | L | F | H | C | K | E | F | H | L | C | D | C | H | S | M | P | V | L | C | D | A | E |
| AP3 | R | Q | V | T | Y | S | K | R | R | M | G | L | F | K | K | A | H | E | L | T | V | F | C | D | A | R |
| AP1 | R | Q | V | T | F | S | K | R | R | A | G | L | L | K | K | A | H | E | I | S | V | L | C | D | A | E |
| **Pm6** | R | * | V | M | F | S | K | R | R | M | G | L | L | K | K | A | Q | E | L | F | V | F | C | D | A | E |
| **Pm1** | R | Q | V | T | F | S | K | R | R | M | G | L | L | K | K | A | Q | E | L | S | V | L | C | D | A | E |
| **Pm2** | R | Q | V | T | F | S | K | R | R | N | G | L | L | K | K | A | Y | E | L | S | V | L | C | D | A | E |
| **Pm3** | R | Q | V | T | F | S | K | R | R | N | G | L | L | K | K | A | Y | E | L | S | V | L | C | D | A | E |
| DAL-1 | R | Q | V | T | F | S | K | R | R | N | G | L | L | K | K | A | Y | E | L | S | V | L | C | D | A | E |
| DAL-3 | R | Q | V | T | F | S | K | R | R | N | G | L | L | K | K | A | Y | E | L | S | V | L | C | D | A | E |
| AGL-6 | R | Q | V | T | F | S | K | R | R | N | G | L | L | K | K | A | Y | E | L | S | V | L | C | D | A | E |
| **Pm4** | R | Q | V | T | F | C | K | R | R | N | G | L | L | K | K | A | Y | E | L | S | V | L | C | D | A | E |
| **Pm13** | R | Q | V | T | F | C | K | R | R | N | G | L | L | K | K | A | Y | E | L | S | V | L | C | D | A | E |
| DAL-2 | R | Q | V | T | F | C | K | R | R | N | G | L | L | K | K | A | Y | E | L | S | V | L | C | D | A | E |
| AG | R | Q | V | T | F | C | K | R | R | N | G | L | L | K | K | A | Y | E | L | S | V | L | C | D | A | E |
| AGL-1 | R | Q | V | T | F | C | K | R | R | N | G | L | L | K | K | A | Y | E | L | S | V | L | C | D | A | E |
| AGL-5 | R | Q | V | T | F | C | K | R | R | N | G | L | L | K | K | A | Y | E | L | S | V | L | C | D | A | E |
| AGL-2 | R | Q | V | T | F | A | K | R | R | N | G | L | L | K | K | A | Y | E | L | S | V | L | C | D | A | E |
| AGL-4 | R | Q | V | T | F | A | K | R | R | N | G | L | L | K | K | A | Y | E | L | S | V | L | C | D | A | E |
| FBP2 | R | Q | V | T | F | A | K | R | R | N | G | L | L | K | K | A | Y | E | L | S | V | L | C | D | A | E |
| OsMADS1 | R | Q | V | T | F | A | K | R | R | N | G | L | L | K | K | A | Y | E | L | S | L | L | C | D | A | E |
| OM1 | R | Q | V | T | F | A | K | R | R | K | R | L | L | K | K | A | Y | E | L | S | V | L | C | D | A | E |

Fig 4.12. An alignment of the deduced amino acid sequences of seven PCR clones (**Pm1, Pm2, Pm3, Pm4, Pm6, Pm7 and Pm13**) of *Pinus radiata* with deduced amino acid sequences of conserved MADS-box regions from various plant species is shown in this figure. The deduced amino acid sequences of seven DNA sequences isolated from *Pinus radiata* were determined using the TRANSLATE programme of GCG. AG, AGL1-6, AP1, and AP3 are from *Arabidopsis thaliana*, OM1 is from *Aranda deborah*, FBP2 is from *Petunia hybrida*, DAL1, DAL2 and DAL3 are from *Picea abies*, OsMADS1 is from *Oryza sativa*. **Blue** highlighted regions are primer regions, residues different from *AGAMOUS* sequence are highlighted **cyan** in primer regions. Residues identical to the deduced amino acid sequence of AG between two primers are highlighted **green**, residues different from AG at this region are highlighted **red**.

The deduced amino acid sequences of Pm4 and Pm13 from *Pinus radiata* together with DAL2 from *Picea abies* share 100% identity to *AGAMOUS* at this conserved region. Pm2 and Pm3 together with *DAL*1 and *DAL*3 from *Picea abies* only have one different amino acid from *AGAMOUS*, by substitution of Serine (S) instead of Cystine (C), indicating that Pm2, Pm3, *DAL*1, and *DAL*3 are not only probably part of a large MADS-box gene family but also close to each other, having the same origin, that is within a conifer grouping. Pm1 and Pm6 also shared a higher percentage identity to *AGAMOUS* at amino acid level than that at nucleotide sequence level, indicating that they are probably also part of the MADS-box gene family. Pm7 showed its distinguishing different amino acid sequence from any other MADS-box amino acid sequences, clearly excluding it from being a member of the MADS-box gene family.

# 4.4 DISCUSSION

## 4. 4.1. DNA ISOLATION FROM *Pinus radiata*

*Pinus radiata* is the most important softwood conifer for commercial forestry production in New Zealand. There is great interest in improving the characteristics of *Pinus radiata*, for example by reducing tree taper and branching, or producing straight logs with low knot blemish (Zobel, 1977). Investigating the genetic control of reproductive sterility has also been attempted in conifer species in recent years (Strauss *et al*, 1995).

The genes from *P. radiata* that give rise to these commercially desirable characteristics of a softwood are usually found in low- or single-copy number (Graham, 1993) in a genome that is seven times larger than the human genome (Neale and Williams, 1991). With long sexual generation intervals, the improvement of these commercially desirable characteristics by conventional breeding programmes has generally been slow, inefficient and subject to random expression of undesirable characteristics (Graham, 1993). Because of the major contribution of conifers to the forest industry, the techniques of molecular biology are being applied to manipulate characteristics that have been difficult or impossible to improve by conventional methods.

To achieve these goals, DNA polymorphisms have to be identified in conifers. The DNA polymorphisms have to be genetic and not artefactual, and the DNA itself must be isolated such that it is free of either mechanical or enzymatic degradation; and has the quality and amount appropriate for PCR, cloning and Southern blotting. Several researchers' work has demonstrated that meeting these requirements with woody conifer species has been far more difficult than with annuals or biennials (Couch and FritZ, 1990; Howland *et al.*, 1991). Needles of conifer species, which are a common source of DNA, contain large amounts of polysaccharides and secondary metabolites, such as polyphenols, terpenoids, and resins (Ziegenhagen, *et al.*, 1993). The phenolic groups that were found in high concentration in conifers are oxidised in the presence of polyphenol oxidases to form insoluble complexes with nucleic acids (Katterman and Shattuck, 1983). This effect was also observed during genomic DNA extraction from *P. radiata* by Graham (1993). This effect made it very difficult to isolate pure DNA with a high yield. Polysaccharide contamination is the most common problem affecting plant DNA purity as pointed out by Murray and Thompson (1980). The carbohydrates can inhibit the activity of many

molecular biological enzymes, such as polymerase, ligases and restriction endonucleases (Aoki and Koshihara, 1972; Furukawa and Bhavadna, 1983; Richards, 1988; Shioda and Marakami-muofushi, 1987), and can interfere with concentrating the DNA sample (Fang, *et al.*, 1992). For *Pinus* tissue, which is rich in both polysaccharides and polyphenols, the usual procedures for isolating high quality DNA with a high yield generally fail, although several methods have been published [eg. Alosi *et al.*, (1990), Manning (1991), Couch and Fritz (1990), and Tulsieram *et al.*, (1992)].

I chose a modified CTAB procedure developed by Sutton (personal communication) at B.C. Forest Research Station, B.C. Canada. This technique capitalised on the previous observations that nucleic acids can be selectively precipitated with CTAB and that DNA is soluble in CTAB + 0.7 M NaCl, whilst many polysaccharides are insoluble at this salt concentration (Murray and Thompson, 1980). Thus the nucleic acids and polysaccharides can be separated from each other. Murray and Thompson did not detect any evidence of polyphenol contamination in the extracted DNA solution when the CTAB procedure was applied, and they inferred that polyphenoloxidase activity was inhibited through this procedure (Murray and Thompson, 1980). An even higher concentration of salt (1.4M NaCl) in the CTAB buffer helps further to remove polysaccharides whilst maintaining the DNA in solution (Fang *et al.*, 1992) and it was this modified procedure that I chose to use. Even after this procedure, I found that it was usually helpful if an extra round of precipitation with 0.3 M sodium acetate and ethanol was applied to ensure a pure DNA product as assessed by restriction digestion. With this method, the present study has consistently obtained DNA of a satisfactory purity with $OD_{260}/OD_{280}$ values of 1.7 -1.96, and a restrictable DNA from *Pinus radiata*, as shown in Fig 4.1. The pine genomic DNA precipitated from TE buffer was only partially digested, whereas the pine genomic DNA treated with another round of ethanol precipitation was digested completely. This result is in agreement with the observation made by Fang *et al.*, (1992). They interpreted this difference in the digestibility of DNA as being caused by the polysaccharide contamination. The activity of restriction endonucleases were inhibited by these carbohydrates. By using CTAB precipitation procedure in conjunction with an extra round of ethanol treatment has been demonstrated as an efficient method of retaining a high quality genomic DNA from *Pinus* tissue rich in polysaccharides and polyphenols in this study.

## 4.4.2. HYBRIDISATION WITH PINE GENOMIC DNA, USING A HETEROLOGOUS PROBE.

The Southern blot approach, searching for *Pinus* sequences, which are similar to the *Arabidopsis AGAMOUS* gene has been a challenging task in this study. The evolutionary relationship between these two species is rather distant. However, the *AG* gene contains a evolutionary conserved MADS-box region, which was assumed to be conserved in *P. radiata.* In addition, the copy number of genes containing a MADS-box region is speculated to be low, and the size of the *Pinus* genome is very large (seven times larger than the human genome, Neale and Williams, 1991). These factors made this Southern blot study a difficult task to fulfil.

To ensure sufficient pine genomic DNA to hybridise with the heterologous probe (*AGAMOUS* cDNA from *Arabidopsis*), 35 μg pine DNA digested with two restriction endonucleases, *Eco* RI and *Bam* HI was loaded onto each lane (Fig 4.3). To ensure an efficient transfer of the digested pine DNA from an agarose gel to the Nylon membrane during the Southern (capillary) blotting, a depurination procedure prior to denaturation of the agarose gel was applied. The agarose gel was treated with 250 mM HCl for two times, each time allowed 15 minutes. This step was to ensure a complete transfer of large DNA fragments from the agarose gel to the Nylon membrane.

Southern blot study showed the hybridisation result under relatively low stringency condition, in which both hybridisation and washing were performed at $60^0$C. The tentative evidence of hybridisation between pine genomic DNA and the *AGAMOUS* cDNA probe with the least background interference was shown (Fig 4.4).

It need to be acknowledged that this Southern blot study did produce a puzzling result: In Fig 4.4, lane 2 and lane 3 displayed an almost identical three bands, even though pine genomic DNA was digested with two different restriction enzymes in two lanes. The causes for this ambiguity was not clear. But two hybridising bands (4.7 kb and 2.8 kb in lane 2) and two hybridising bands (4.7 kb and 4.0 kb in lane 3) still remained after the blot was washed under a high stringency condition, suggesting a possible hybridisation between *AG* cDNA and pine genomic DNA.

It has to be emphasised that the hybridised signals from this present study are rather weak, probably due to the low copy number of the targeted DNA sequences in a large *Pinus*

genome. There were also a couple of vaguely visible bands in the background in this figure, but when the same Southern blot was washed at a higher temperature ($65^0$C), they disappeared. They were more likely to be products of unspecific bindings in this Southern blot study. Nevertheless, the tentative evidence of hybridising between *AG* cDNA probe and pine genomic DNA suggested the possible presence of MAD-box related genes in *Pinus radiata.* There were also some non-specific bindings between the probe, λ DNA standard markers (lanes 1,10 Fig 4.4) and plasmid DNA vector for *LFY* insert (pBluescript) (lanes 6,7 Fig 4.4) and *AG* insert [pGEM7Zf(+)] (lanes 8,9 Fig 4.4). This was probably due to the fact that the *AG* cDNA probe might contain some vector DNA, and they hybridised with λ DNA markers and the vector DNAs and produced strong hybridising signals.

Southern blot approach, using a heterologous probe has also been attempted in C. Walter's group at New Zealand Forest Research Insititute. They had experienced some difficulties in finding meaningful hybridising signals between *LEAFY* cDNA probe and pine genomic DNA (personal communication). It seems that Southern blot study probably is not the best strategy to search for genes with a low copy number in a large *Pinus* genome.

In order to clarify this Southern study result, detecting the presence of MADS-box related genes in *Pinus radiata*, a PCR-based approach was used to amplify DNA sequences related to MADS-box genes from *Pinus radiata*.

## 4. 4.3. ISOLATING *PINUS* DNA SEQUENCES RELATED TO MADS-BOX GENES BY PCR

Isolating DNA sequences related to MADS-box genes by PCR has been demonstrated in birch (*Betula pendula*) (Tikka *et al.*, 1993) and black spruce (*Picea mariana*) (Rutledge *et al.*, 1993). By using degenerate oligonucleotides corresponding to two well-conserved sequences in the MADS-box region, they amplified by PCR approximately 95 bp and 60 bp regions of DNA sequences from birch and black spruce genomic DNA respectively. The amplified products have been identified as members of the MADS-box gene family.

The essential part of this technique is to design a set of degenerate primers. These primers have to bind to the template genomic DNA from *Pinus* to allow the amplification of the required DNA region. At the same time, it needs to be ensured that the primers do not bind non specifically to other DNA sequences in the genome. A set of degenerate primers

was designed according to Tikka *et al* (1993), targeting the conserved region between RQVT and VLCDAE within the MADS-box domain. RQVT is a conserved phosphorylation site which was recommended to be used to design degenerate oligonucleotide sequence by Ma *et al.*, (1991).

It was found that the amplification of pine MADS-box DNA sequences by PCR did not work at the annealing temperature of $55^0$C. It required a relatively low annealing temperature (37-$45^0$C), even when using primers targeting a highly conserved region in this study. A hot start in this reaction was also necessary, and a sufficient primer concentration in the PCR mixture was crucial to allow a successful amplification of the targeted DNA fragments (Fig 4.5). Lanes 4 and 5 in Fig 4.5 show visible bands after the primer concentration was increased two fold higher than that in lane 3, where there no bands were detectable (Fig 4.5). The lower annealing temperature, which was required and sufficient primer concentration would be an indication of the difficulties to amplify a rare number of copies of MADS-box genes in a large *Pinus* genome.

In order to obtain enough MADS-box DNA fragments, PCR products shown in Fig 4.5 from the first round were reamplified for another round, which resulted in a considerable increase in yield, as shown in Fig 4.6.

This exercise inevitably brings up the issue of error rates during this PCR based amplification. Saiki *et al* (1988) assessed the fidelity of the thermostable *Taq* polymerase in the amplification reaction by cloning and sequencing individual amplification products. They reported that an overall error frequency was 0.25%, and if consistant over the 30 PCR cycles, the misincorporation rate per nucleotide per cycle for *Taq* polymerase was estimated at 2 x $10^{-4}$ (Saiki *et al*,. 1988). Sambrook *et al* (1989) also pointed out that such occasional errors were not a problem when the products of the entire amplification reaction were used as hybridization probes or as templates for direct DNA sequencing.

In this present study, the total length of the amplified product is 95 bp, including the restriction sites at both ends. Even though, the total amplification cycle is about 80 cycles, but if it is consistant over the 80 cycles, the error frquency could be calculated using the formular m = 2(f/d) by Saiki *et al* (1988) (m: misincorporation rate per nucleotide per cycle, 2 x $10^{-4}$ , d: the number of doublings). The overall error frequency is 0.8% in this study calculated according to this formula, which is still very low. So the chances of

getting misleading DNA sequencing data due to the error rate of the DNA amplification by PCR are minimal.

There are some extra bands with different molecular weights revealed in Fig 4.6 and Fig 4.7. these bands probably were the result of hybridisation between degenerate primers and the template pine genomic DNA sequences, which may or may not be related to MADS-box genes in the pine genome. Because they all have different numbers of nucleotides from the targeted MADS-box region, and the sequence of these DNA fragments were not available, it is difficult to determine whether they are related to MADS-box genes in the *Pinus* genome or not.

## 4. 4.4. ANALYSIS OF DNA SEQUENCES ISOLATED FROM *Pinus radiata* BY PCR

Since a mixed population of PCR products from a family of MADS-box genes was expected, it was necessary to clone individual products prior to sequencing. PCR amplified pine DNA fragments were cloned into M13mp18.

M13 was chosen as a subcloning vector for dideoxy sequencing because M13 bacteriophages are packaged single strand DNA which are extruded from infected *Escherichia coli* cells into the surrounding culture medium. This means that considerable quantities of single stranded template DNA can be easily produced. Another important reason is that the M13mp vectors have a quick colour assay to identify bacterial cells infected with phage containing an insert.

The M13 mp series of vectors was constructed by insertion of a restriction fragments of the *E. coli lac* regulatory region into wild-type M13. This fragment contains the region coding for the first 145 amino acids of the α-peptide of the β-galactosidase gene. Within this region synthetic oligonucleotides, containing several unique restriction enzyme sites, have been introduced such that the α-peptide reading frame is retained. When these phages infect defective *E. coli* (F',∇*lac pro* ), which have a deletion within this region of the β-galactosidase gene, complementation occurs and a functional β-galactosidase is produced. In the presence of IPTG (Isopropyl thiogalactoside), the substrate X-gal is hydrolysed by β-galactosidase to bromochloroindole, which confers a blue colour to the infected plaque on a bacterial lawn. If, however, an insert is cloned into one of the synthetic oligonucleotide restriction sites such as to interrupt the α-peptide coding region,

no functional β-galactosidase is produced, the Xgal is not hydrolysed, and the infected plaque remains colourless. This makes detection of recombinants very simple.

Another reason to choose M13mp18 is because that the M13mp18 vector provides several unique restriction sites for cloning (section 4.2.1.22.)

Restriction sites (*Eco* RI and *Xba* I) attached to the end of forward and reverse primers, used in the amplification of pine DNA by PCR in this study were designed specifically to suit for subcloning into the M13mp18 vector.

Having proper restriction sides not only makes it easy for subcloning into the right vector, but also was helpful in judging the reliability of the result sequence on the PAGE sequencing gel. Fourteen clones showed both restriction sites clearly at both ends of the amplified products, and the distance between these two restriction sites was also the expected length according to the *Arabidopsis AGAMOUS* MADS-box sequence. These sequences were identified as probably part of the MADS-box related genes with the exception of Pm7 and Pm12. The nature of these two DNA sequences will be discussed later. One recombinant clone (Pm15, not shown in Fig 4.9) had the expected restriction sites at both ends, but the insert was about nine base pairs longer than the targeted DNA sequence. Comparing its sequence with other amplified sequences did not show any similarity, so this sequence was unlikely to be related to MADS-box genes and was omitted from this study.

Fourteen amplified DNA sequences from *Pinus radiata* were aligned with the conserved MADS-box region of *AGAMOUS* (*AG*) gene from *Arabidopsis*, as shown in Fig 4.9. The sequences showed a different degree of identity to *AG* MADS-box region with a percentage identity to *AG* ranging from 28.9%-84.4% with primer region excluded. Wherever different nucleotides occurred, in comparison with the MADS-box sequence of *AG* from *Arabidopsis*, they usually occurred at the third codon (Fig 4.9).

This result clarified several points. The first one is that the 14 DNA sequences amplified have high degree of identity to *AG* MADS-box. It is therefore concluded that most of them probably are related to pine MADS-box genes, and they are evolutionary conserved in nature. These sequences are not uniformly identical to each other among themselves and they do not have 100% identity to the *AG* MADS-box sequence. This eliminates the possibility of these amplified DNA sequences being contaminated by *Arabidopsis* DNA

during PCR, which is a concern when *Arabidopsis* DNA was consistently used as a positive control during the amplification of pine genomic DNA by PCR.

Secondly the regular occurrence of nucleotides different from the *AG* sequence at the third codon among the 14 amplified pine DNA sequences suggested that the observed differences in DNA sequence were not all due to random incorporation errors during PCR amplification but reflect genuine sequence differences present in the original pine template DNA.

In comparing the amplified DNA sequences with one another, it was found that Pm1, Pm5, Pm10 and Pm14 were almost identical to each other with minor differences only within the primer regions. Pm2, Pm8, Pm9 and Pm11 were also almost identical to each other with minor differences only within primer regions. Pm3, Pm4, Pm6 and Pm13, were each different from another by one or more nucleotides in the amplified region between the two primers. Pm7 and Pm12 were almost identical to each other with minor differences within primer regions. As primers used in this PCR based amplification are degenerate, the amplified sequences at the primer region do not accurately represent the original pine template DNA sequence information at this region, so the DNA sequences isolated in this study were divided into seven different groups based on their sequences excluding primer regions. They are represented by Pm1, Pm2, Pm3, Pm4, Pm6, Pm7 and Pm13. Further characterisation of the DNA sequences did not include primer regions.

## 4. 4.5. CHARACTERISATION OF THE RELATIONSHIP BETWEEN PINE DNA SEQUENCES WITH OTHER MAD-BOX DNA SEQUENCES.

Seven amplified DNA sequences excluding primer regions from *Pinus radiata* and the corresponding regions of eighteen other MADS-box DNA sequences were displayed by the PRETTY program of the GCG, shown in Fig 4.10. PRETTY printed sequences with their columns aligned and displayed a consensus for the alignment to reveal the relationships among these sequences. The consensus was determined by finding the symbol in the column for which its comparison to all of the symbols in the column yielded the greatest number of votes.

Based on this analysis, these seven DNA sequences can be divided into four groups, Pm1 and Pm6 share a similar sequence, Pm2 and Pm3 share a second similar sequence, Pm4 and Pm13 share a third similar sequence and Pm7 is different from all the other MADS-

box DNA sequences. Pm1 and Pm6 show some degrees of identity to other MADS-box genes but clearly not as high as the other four sequences, while Pm7 shows a rather low identity to all listed MADS-box genes.

To further interpret this result, a table (Table **4.3**) was made to show the percentage identity between the seven pine DNA sequences with sequences of eight MADS-box genes from *Arabidopsis*. Pm2 and Pm3 show a very high identity to DNA sequences of *AG, AGL-5, AGL-4,* and *AGL-6* genes, ranging from 80% to 84.4%. Pm4 and Pm13 show a very high identity to DNA sequences of *AG* and *AGL-5*, ranging from 82.2% to 84.4%, but show a relatively lower identity to AGL-4 and AGL-6 at 73.3% and 75.5%. Pm2, Pm3, Pm4 and Pm13 all show a lower identity to *AGL*-2, *AGL*-1, and *AP3*, ranging from 71.1% to 77.8%, but they show their lowest identity to *AP*1 at 64.4% and 68.9%. Pm1 and Pm6 show their identity to these eight *Arabidopsis* MADS-box genes at almost 20% lower, ranging from 51.1% to 66.7%. They also show their lowest percentage identity to *AP*1 at 51.1% and 55.6%.

Pm7 generally shows a very low identity to all of the eight selected genes from *Arabidopsis*, ranging from 28.9% to 35.6%. The deduced amino acid sequence shown in Fig 4.12 is almost completely different from the listed MADS-box genes. The possibility of Pm7 being part of a MADS-box gene is very limited. The reason why this sequence was isolated by PCR from the pine genome was probably due to the use of degenerate primers, and a low annealing temperature, leading to amplification of DNA sequences unrelated to MADS-box genes. (sequence data shown in Fig 4.9).

To further characterise the relationship of the remaining six pine DNA sequences among themselves and their relationship with other MADS-box genes, a dendrogram tree based on the PILEUP program of the GCG package was plotted to show the clustering relationships of these sequences (Fig 4.11). PILEUP creates a multiple sequence alignment using a simplification of the progressive alignment method of Feng and Doolittle (1987). The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster can then be aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences can be aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments that include increasingly dissimilar sequences

and clusters, until all sequences have been included in the final pairwise alignment. A dendrogram tree is a representation of clustering relationships, showing the order of the pairwise alignments of the selected DNA sequences. The clustering strategy represented by the dendrogram is called UPGMA (Unweighted Pair-Group Method Using Arithmetic averages) (Sneath and Sokal, 1973). This clustering method is sensitive to the order in which sequences are aligned. A clustering algorithm determines this order from the pairwise similarities calculated before the final alignments are done (PILEUP, Multiple Sequence analysis Section of the GCG Package, Version 7, April 1991). According to PILEUP, the distance along the horizontal axis is proportional to the difference between clusters and sequences in Fig 4.11, the vertical axis has no significance at all.

In Fig 4.11, a close relationship between Pm2, Pm3, Pm4 and Pm13 is clearly shown, and they are shown to form a *Pinus* cluster ; *DAL-1* and *DAL-3* are closely related; they form a *Picea* cluster. The *Pinus* and *Picea* cluster together to form a conifer group based on their MADS-box DNA sequences shown in box **III**. The immediate neighbouring group is an angiosperm group. It is made up of four MADS-box genes (*AG*, and three *AG*-like genes) from two dicotyledon species, *Arabidopsis thaliana* and *Brassica napus* of *Brassicaceae* family shown in box **II**. The next neighbouring group to these two closely related groups is a monocotyledon group. It is made up of four MADS-box genes from three monocotyledon species of two families, Poaceae and Orchidaceae shown in box **I**.

The group next to these three groups of MADS-box related DNA sequences is made up of four genes from *Arabidopsis thaliana* and one gene from *Petunia hybrida*. *DAL-2* and *TAG1* form a cluster next to these four groups of MADS-box related genes.

Even though *AP*1 is a member of the MADS-box gene family, it is alone separated from almost all the rest of the DNA groups listed in this dendrogram, clearly showing its significantly different DNA sequences from other MADS-box genes. Pm1 and Pm6 are closely related to each other shown in this dendrogram, and they form a cluster which is separated from all the listed MADS-box DNA sequences from plant species, indicating a more distant relationship with other MADS-box genes used in the alignment. *DMU* is a MADS-box gene from *Drosophilia melanogaster*, which is clearly separated from all the plant MADS-box genes shown in this dendrogram, suggesting its distant relationship with its plant counterparts based on sequence comparison of MADS-box genes.

The comparison of the potential expression products of the seven pine DNA sequences (Pm1-4, Pm6, Pm7, and Pm13) with other major MADS-box genes further confirmed the close relatedness of some of the pine DNA sequences to these MADS-box genes. The comparison of the deduced amino acid sequence of seven pine DNA sequences and fourteen major MADS-box genes presented in Fig 4.12 shows that Pm4, Pm13 and DAL-2 [a MADS-box gene isolated from *Picea abies* (Tandre *et al.*, 1995)] share identical amino acid sequences to AG, AGL-1 and AGL-5 of *Arabidopsis* in the region under consideration. Pm2 and Pm3 together with DAL-1 and DAL-3 [two MADS-box genes isolated from *Picea abies* (Tandre *et al.*, 1995)] share identical amino acid sequence to AGL-6. Pm2 and Pm3 are only different from AG, AGL-1 and AGL-5 at one amino acid by substitution of Serine (S) instead of Cysteine (C). It is of interest that AGL-2, AGL-4 also only differ from AG, AGL-1, and AGL-5 at the same one amino acid position, having an Alanine (A) instead of a Cysteine (C). Due to differences in function of these groups of MADS-box genes and the importance of Cysteine residues in determining protein folding (Lehninger, 1975), it is hypothesised that these changes may have functional significance. Pm1 is different from AG by three amino acids, Pm6 is different from AG by four amino acids, excluding primer sequences. The positions at which Pm1 and Pm6 differ from the AG, AGL-1, and AGL-5 group are similar to the positions at which the *Arabidopsis* AP3 and AP1 (genes controlling the earlier stages of the floral development) differ from the AG, AGL-1, and AGL-5 group, which control the late stage of the floral development. Since, at least in the case of AP3 and Pm6 there are some common amino acids at sites which differ from the AG, AGL-1, and AGL-5 group, it is tempting to speculate that Pm6 and Pm1 may likewise have the same function as AP3 or AP1 and may be involved in the earlier stages of floral development. Pm7 again shows an almost completely different amino acid sequence from all the rest of the MADS-box gene products, so it is unlikely that Pm7 is part of a MADS-box gene from *Pinus radiata.* As Pm12 is almost identical to Pm7 in nucleotide sequence, Pm12 is not expected to be related to MADS-box genes either.

It is important to point out that the difference in nucleotide sequences between Pm1 and Pm6 is only by one nucleotide, so are the differences between Pm2 and Pm3, and between Pm4 and Pm13 (Fig 4.9). This one base difference between the members of a pair could possibly be due to the misincorporation during the amplification by PCR. If we ignore this

difference possibly caused by PCR errors, the twelve DNA sequences isolated from *Pinus radiata* related to MADS-box genes can be divided into three groups. Group 1 includes Pm1, Pm6, Pm10, Pm5, and Pm14. Group 2 includes Pm2, Pm3, Pm8, Pm9, and Pm11. Group 3 includes Pm4 and Pm13.

From the combined results of the comparison of the DNA sequence data and the deduced amino acid sequence data, it is concluded that three groups of DNA sequences isolated from *Pinus radiata*, represented by Pm1, Pm2 and Pm4 are part of *Pinus* homologues to genes that control floral development in angiosperms.

Recent studies have identified some MADS-box genes as early-acting genes that promote the formation of floral meristems and some MADS-box genes as later-acting genes that determine the fate of floral organ primordia in *Arabidopsis*. The process of flower development is a process of the regulatory interactions between early-acting genes and late-acting genes, and the regulatory interactions between the organ identity genes.

*AP*1 is an early-acting gene, it acts locally to specify the identity of floral meristems to define pedicel tissue as floral rather than inflorescence. It is also involved in specifying the identity of the two outer whorls of organs, sepals and petals (Mandel, *et al* 1992a). In Yanofsky's (1995) model of homeotic organ identity genes which control three different activities (designated by Coen and Meyerowitz A, B, and C) to specify the four different organ types. *AP*1 from *Arabidopsis* specifies the identity of sepals and petals, and is designated as a class A gene. *AP*3 is a later-acting gene, defining the organ identity of petals and stamens (Jack *et al.,* 1992). According to Yanofsky's model, *AP*3 is a class B gene. *AG* is a late-acting gene, defining the organ identity of stamens and carpels (Yanofsky, *et al.*, 1990) and *AG* is a class C gene in this model. *AGL*-1 and *AGL*-5 are also late-acting genes, *AGL*-1 is only expressed in carpels, particularly in ovules but not in stamens (Ma *et al* ., 1991). *AGL*-5 requires *AG* for expression and its RNA begins to accumulate shortly after that of *AG*, Savidge and Yanofsky speculated that *AGL*-5 is a candidate target gene for *AG* (Savidge et al., 1995).

The comparison between DNA sequences isolated from *Pinus radiata* and MADS-box genes from angiosperm species shows that group 2 (Pm2, Pm3, Pm8, Pm9, and Pm11) and group 3 (Pm4 and Pm13) pine DNA sequences are more closely related to MADS-box genes controlling late floral development, such as *AG* from *Arabidopsis thaliana* and those

*AGAMOUS*-like genes from a number of other plant species. Their close relatedness to *DAL-1,2,3* genes from *Picea abies* (Tandre *et al.*, 1995) not only confirms their common coniferous origin but also suggests that these *P. radiata* DNA sequences may belong to MADS-box genes which have a similar function compared to *DAL-1,2,3* genes. *DAL-1,2,3* genes from *P. abies* have been found involved in the development of both male and female reproductive organs, with *DAL-2* only being expressed in developing male and female cones.

As Pm2, Pm3, Pm4 and Pm13 were placed right next to *DAL-1, 3*, and *AG*-like MADS-box genes on the dendrogram tree, and their deduced amino acid sequences were very similar to each other, it is hypothesised that group 2 pine DNA sequences (Pm2, Pm3, Pm8, Pm9, and Pm11) and group 3 pine DNA sequences (Pm4 and Pm13) probably belong to genes which are functional as well as structural homologues of the angiosperm class C genes. The Gene Transformation and Gene Expression Group at New Zealand Forest Research Institute headed by C. Walter is currently using Pm1, Pm2 and Pm4 sequences as probes to screen a cDNA library constructed from the male cone tissue of *Pinus radiata* aiming at identifying and characterising proposed class C and class B floral organ specific genes from *Pinus radiata* (personal communication).

Even though group 1 DNA sequences (Pm1, Pm5, Pm6, Pm10 and Pm14) represented by Pm1 and Pm6 have a slightly lower percentage identity to *Arabidopsis* MADS-box genes than group 2 and group 3 pine DNA sequences, they still have about 60% identity rate to the listed MADS-box genes from *Arabidopsis*. In particular, the percentage identity between Pm1 and *AP3* is well over 60% (Table **4.3**). At the amino acid level, as reviewed before, the positions at which Pm1 and Pm6 differ from the AG, AGL-1, and AGL-5 group are similar to the positions at which the *Arabidopsis* AP3 (class B) and AP1 differ from the AG, AGL-1, and AGL-5 group (class C). At least in the case of AP3 and Pm6, there are some common amino acids at sites which differ from the AG group (Fig 4.12). It is tempting to speculate that group 1 pine DNA sequences (Pm1, Pm5, Pm6, Pm10, and Pm14) are more closely related to homeotic genes controlling the earlier steps of floral development, such as class B genes controlling petal and stamen development in angiosperms. Using this group of DNA sequences as probes could possibly lead to identify homeotic genes controlling the male cone development in *Pinus radiata*.

Further characterisation and manipulation of these MADS-box genes could allow generation of reproductive or male sterility in *Pinus radiata*. The information obtained from this present study of the existence of *Pinus* homologues to genes that control floral development in angiosperms confirms the prediction made by Tandre *et al* (1995) that the control systems for reproductive development in conifers and angiosperms had a common origin in a complex ancestral control system, having evolved by a series of gene duplications, long before the appearance of the hermaphroditic angiosperm flower.

# CHAPTER 5.0 SUMMARY AND CONCLUSIONS

This thesis presents a result of a systematic and extensive study of male cone development in *Pinus radiata.* The morphological, anatomical changes and the timing of these changes during male cone development of *Pinus radiata* growing in the central part of the North island, New Zealand were examined. In correlation with the morphological and anatomical changes described, the appearance of some protein species and isoforms of four key enzymes marking developmental events were recorded. A search for the floral-specific genes controlling these developmental events was also attempted. MADS-box DNA sequences belonging to a homeotic gene family controlling floral development in higher plants have been reported for the first time in the genus, *Pinus* in this study.

The timing of developmental events and their relationship with environmental factors in comparison with pine species growing in the northern hemisphere was discussed. Some significant morphological aspects, and structural/ultrastructrual changes during male cone development in *Pinus radiata* were reported in the morphological and anatomical study.

The first appearance of the potential male cone primordia of *P. radiata* was recorded in early December, which is the early season of the New Zealand summer. The potential (bullet-shaped) male cone primordia were formed in mid January, and microsporophylls were initiated on these primordia in late February, which is in the late New Zealand summer. Unlike northern pine species, the development of microsporophylls and the differentiation of the microsporogenous tissue progress continuously from late February to early July without a dormant break. Meiosis was presumed to occur in late May and to be completed in the beginning of July, as judged by observation of the formation of microspore-tetrads and pollen grains. A flow cytometry study however did not conclusively detect the haploid nuclei in germinated pollen grains. The precise timing of the completion of meiosis then remains in doubt. Pollen was shed in early July, which is in the middle of New Zealand winter. Temperature is believed to be a major environmental factor influencing the pace of male cone development in *Pinus* species.

A morphological phenomenon noticed in this study is that male cones mature at a different "rate", depending on their locations on the male cone bearing shoots. Male

cones collected from the basal region of the male cone bearing shoots were found to have an advanced development over male cones collected from the distal region of the same shoot. Microsporangia in the basal microsporophylls also exhibited a more advanced development over the microsporangia in the distal microsporophylls from the same male cone. Such a variation of the male cone maturation within the shoot and within the cone itself is considered as an advantage for this species, allowing pollination to work more effectively over time.

Structural and ultrastructrual changes of the tapetal cells and pollen mother cells during male cone development, especially during the meiotic process are the focus in this study. When pollen mother cells proceeded into the meiotic process, the tapetal cells underwent some significant structural changes: their cell wall appeared to be thinner, the rough endoplasmic reticulum (RER) became extremely dilated, the cytoplasm became intensely basophilic because of the density in ribosomes which were arranged in polyribosome groups. The relative quantity of plastids and mitochondria was reduced apparently due to degradation by prominent autophagic vacuoles which occurred in the cytoplasm when pollen mother cells were in the late prophase I of meiosis. The intact tapetum layer completely disappeared and the tapetal cells were severely degraded when microspore-tetrads were formed in microsporangia. The hypersecretory feature of the tapetum cells suits their function as a nutritive layer, allowing them to secrete nutrients supplied by the tapetal cells and the middle microsporangial layer cells into the microsporangia locules. Subsequently these nutritive products are available to be taken up by the developing pollen mother cells or microspores.

In the early stages of microsporangia, plasmodesmata were seen among tapetal cells and early sporogenous cells. They were also seen between tapetal and pollen mother cells. This syncytium character of tapetal cells and early sporogenous cells explains why these two cell types develop simultaneously and display similar structural features in the earlier stages of microsporangia.

Plasmodesmata connections were soon blocked by the occurrence of callosic wall material between tapetal cells and microspore mother cells and among pollen mother cells, when pollen mother cells entered the prophase I of meiosis. The exchange of the genetic information via intercellular connections between pollen mother cells and the tapetal cells with a sporophytic origin was undoubtedly terminated, and this allows

pollen mother cells to develop towards meiosis independently and start a gametophyte generation.

When pollen mother cells entered the prophase I of meiosis, the pairing and contraction of chromosomes of pollen mother cells was recorded. When pollen mother cells developed closer towards the reduction division, chromosomes became thick strands and clumped in a tangled mass filling a portion of the nucleus. Cytoplasmic organelles became dedifferentiated. The relative number of plastids and mitochondria decreased due to the degradation by the autophagic vacuoles. The dedifferentiation of these cytoplasmic organelles is considered necessary for diploid pollen mother cells to develop into haploid pollen grains.

It is generally agreed that floral development is a result of temporal and spatial expressions of a series of genes under the influence of environmental factors. The timing and the cellular structural changes of male cone development provided a foundation to understand the genetic mechanism of this process. For example, information obtained from this anatomical study combined with *in situ* hybridisation techniques could make it possible to locate expressions of particular genes at particular stages during male cone development in a specific cellular or subcellular locations.

In correlation with these cytological changes characterised in the morphological and anatomical study of male cone development, changes of the soluble protein content, banding patterns of the total soluble protein, banding patterns of four isoenzymes were studied by SDS-PAGE and isoelectric focusing techniques.

The result of this study showed that the total soluble protein content did not increase with the increase of the male cone size, but instead showed a sharp drop before the shedding of mature pollen grains. SDS-PAGE has shown differences in protein patterns between the vegetative needle tissue and the successive stages of male cone development. A protein species of 20.5 KD in particular has been detected as a potential male cone tissue specific gene expression product. It was only detected in male cone tissues and pollen extracts, but not seen in needle fascicle tissues. Protein species of 31.50 KD and 33.40 KD occurred in all male cone tissues, except the earlier male cone primordia stage. Protein species of 27.80 KD and 28.50 KD occurred in the

male cone tissue after microsporangia fully developed. Protein species of 22.50 KD only occurred in the earlier developmental stages of the male cone before the late meiosis stages started, so did a protein species of 17.40 KD. The 17.40 KD protein was also detected in the mature pollen extract.

Acid phosphatase, esterase, malate dehydrogenase and peroxidase were studied during male cone development, using isoelectric focusing methodology. The enzyme activity and the number of isoforms of each enzyme exhibited variations in the banding pattern, in relation to the different developmental stages of the male cone. In particular, some unique enzyme isoform bands mark very specific stages of development only: isoforms of malate dehydrogenase with pIs 5.70, 6.95, 7.15, 7.25, 7.40 were detected in the early stages of the male cone tissue (the initiation stage of microsporophyll primordia); isoforms of esterase with pIs 5.20 and 5.30 were detected in the stage when microsporangia were just formed in some of the earlier formed microsporophylls; an isoform of peroxidase with pI 4.70 was only detected in the stage when pollen mother cells were at the late prophase I of meiosis; isoforms of acid phosphatase with pIs 5.30, 5.60 and isoforms of peroxidase with pIs 3.20 and 4.15 were only detected in the stage when microspore-tetrad and pollen grains were formed. Isoforms of acid phosphatase with pI 5.0, isoforms of esterase with pIs 6.05 and 7.25 and isoforms of malate dehydrogenase with pIs 5.40 and 5.60 were only detected in pollen extracts. Despite the intense enzyme activity of malate dehydrogenase exhibited in the earlier developmental stages of the male cone tissue and pollen extracts, no signs of any isoform bands of this enzyme were detected in the later developmental stages of the male cone tissue, in which pollen mother cells were in the late prophase I of meiosis.

Protein/enzyme markers unique to certain developmental stages revealed from this study provide us with valuable information about biochemical processes in relation to cellular structural changes in microsporangial tissues during male cone development in *Pinus radiata*.

At the molecular level, a Southern blot study provided some tentative evidences of hybridising beween *AG* cDNA and pine genomic DNA, suggesting the possible existence of MADS-box genes which are likely to be controlling the "flower" development in *Pinus radiata*. PCR techniques applied in this project successfully

isolated three DNA sequences (Pm1, Pm2 and Pm4), which share a nucleotide identity, ranging from 60% to 84.4% (excluding primer regions) to *AGAMOUS*. The deduced amino acid sequence of Pm2 shares 93.3% identity to the deduced amino acid sequence of *AGAMOUS* at the conserved region of the MADS-box with the substitution of Cysteine (C) with Serine (S). The deduced amino acid sequence of PM4 shares 100% identity to the deduced amino acid sequence of *AGAMOUS* gene. At amino acid level, the positions at which Pm1 differ from the AG are similar to the positions at which the *Arabidopsis* AP3 differ from AG. It is speculated that the Pm1 DNA sequence is probably the conserved region of pine MADS-box genes controlling the earlier steps of floral development, analogous to class B genes controlling petal and stamen development in angiosperms. Pm2 and Pm4 are most likely to be the conserved regions of pine MADS-box genes controlling the late steps of floral development, such as class C genes determining the identity of male floral parts (stamens) and female parts (carpels) in angiosperms.

This molecular biology study confirmed the existence of MADS-box genes in the genome of *Pinus radiata*, and DNA fragments amplified by PCR from *Pinus radiata* have been identified as being related to the floral homeotic gene (*AGAMOUS*).

To carry on with this project, the Gene Transformation and Gene Expression Group at New Zealand Forest Research Institute is currently screening the cDNA library constructed from the reproductive tissue of *Pinus radiata* with labelled *Pinus* MADS-box DNA sequences isolated from this study to identify and characterise floral specific cDNAs of *Pinus radiata*. Northern blot and *in situ* hybridisation study is applied to confirm that the isolated cDNA is floral tissue/stage specific. After cloning this floral specific cDNA into a special designed vector in antisense orientation under the control of a strong promoter, the construct could be transformed into embryogenic tissue, using a transformation system developed by Walter *et al* (1994). Transformed somatic embryos would be propagated and pine trees regenerated would be expected to be reproductive sterile, especially male sterile.

Another approach of regulating floral development is isolating and characterising the promoter region of floral specific genes (for example, a homologous gene to *AP3* of *Arabidopsis thaliana*). By combining a cytotoxic gene [for example, the diphtheria toxin A chain coding sequence, (DTA)] with this floral organ-specific gene promoter, the

particular floral organ could be genetically ablated, so that reproductive sterility or male sterility could be achieved. This approach is also under investigation by the group at NZFRI.

These applied aspects of the thesis work described here remain an objective of many forestry biotechnology laboratories world-wide. The achievement of these aims would result in large scale economic gains in plantation forestry.

In conclusion this present study investigated the fundamental biological events in male cone development and laid down the foundation for further characterisation and manipulation of reproductive-specific genes, which could allow generation of reproductive or male sterility in *Pinus radiata*.

# APPENDIX 1. A PRELIMINARY PLOIDY STUDY OF MALE CONE DEVELOPMENT IN *Pinus radiata* BY FLOW CYTOMETRY - IN COLLABORATION WITH M.E. HOPPING AT CYTOMETRY SERVICES, WAIKANAE, NEW ZEALAND.

## Aims and Methods

It is hypothesised that nuclei from sporogenous tissues of *Pinus radiata* at times prior to meiosis would maintain about the same ratio for percent nuclei in G0/G1, S-phase and G2; As the microspore mother cells enter prophase, the percent nuclei in S-phase would be expected to increase and haploid nuclei would appear as meiosis ends.

According to the results of the anatomical study by light and electron microscopy, sporogenous cells of the male cone tissues collected on 19/4/92 were assessed as being at times prior to meiosis. Sporogenous cells of the male cone tissues collected on 20/5/92 showed characteristics of entry into the meiosis stage. Well formed microspore-tetrads and pollen grains were seen in the male cone tissues collected on 2/7/92. Meiosis was therefore assumed to have been completed by late June.

A flow cytometry study was designed to confirm these microscopy results with a ploidy data analysus of nuclei taken from tissues at each stage.

Dividing cells always pass through a regular cell cycle. Commonly, the cell cycle is divided into interphase and the four phases of mitosis. Interphase is the phase between successive mitotic divisions. It is also called "resting phase" which is the stage most cells were at when the flow cytometric detection was performed. Interphase is divided into three periods, which are designated G0/G1, S, and G2. The G0/G1 period occurs after mitosis and is primarily a time of growth of the cytoplasmic material, including the various organelles. The genetic material (DNA) is not duplicated in this period, but starts later in the S period for the next round of mitosis. The determination of the ploidy level for the normal 2n tissue cell then is generally based on the nuclei ploidy at the G0/G1 period.

Nuclei from microsporangia of male cone tissue collected on 19/4/92, 20/5/92, 16/6/92, 2/7/92 and germinated pollen tubes of *Pinus radiata* were released from plant tissues by

chopping them in a specially designed neutral buffer (Galbraith *et al.*, 1983), and then they were stained with a DNA-specific fluorescent dye. PI (Propidium iodide) was used as a fluorescent dye in this experiment. The cellular and subcellular structure of these selected male cone tissues are illustrated in Figs 2.9, 2.10 for tissues collected on 19/4/92; Figs 2.11, 2.12, 2.21, 2.22, 2.23, 2.24 for tissues collected on 20/5/92; Figs 2.34, 2.35 for tissues collected on 16/6/92 and Figs 2.15, 2.16, 2.17 for tissues collected on 2/7/92. These figures are presented in Chapter Two of this thesis.

These stained individual nuclei were analysed on an EPICS Profile II flow cytometer (Coulter Electronics Inc., U.S.A.) fitted with a Cyonics argon laser (488nm) operating at 15mW. Sheath fluid consisted of 600mg/l NaCl and 6 mg/l Triton X-100 in distilled water and was filtered (0.2 μm) before use. Nuclei samples were analysed at flow rates of 11-20μl/min and sheath pressures of 72.3 kPa.

The stained individual nuclei were passed through the cytometer flow cell where they were illuminated at an excitation wavelength at 635nm. Emitted fluorescence yielded information on the DNA content of individual nuclei. Fluorescence that exceeded 635 nm was collected and results displayed as single parameter histograms of number of nuclei in each 1023 channels.

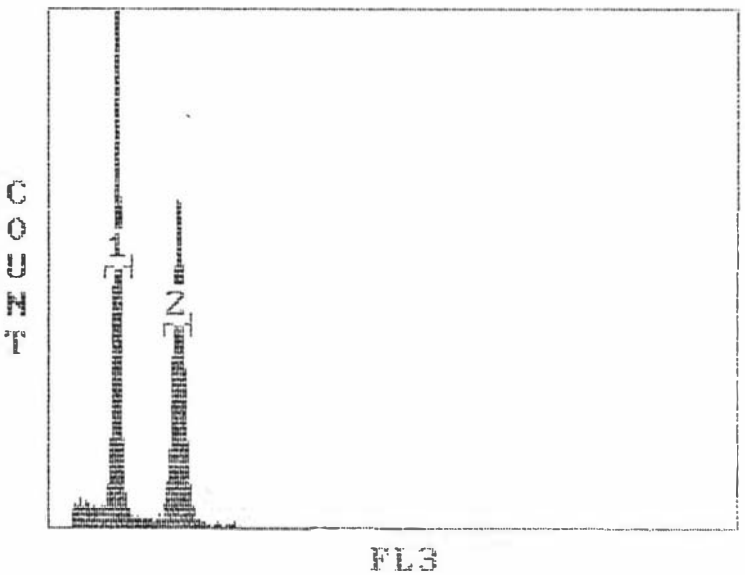
Samples of PI (Propidium iodide) alone were run prior to sample introduction to minimise dye loss to tubing, and data from the first 20s of run time were discarded to avoid instrument instability following sample introduction.

An aliquot (20 μl) of Immuno Check fluorospheres (Coulter Electronics Inc.) was added to most samples before use. Minimum (MIN), maximum (MAX) and mean (MEAN) channel numbers of G0/G1 nuclei, G0/G1 nuclei count (COUNT), half peak height coefficients of variation (HPCV) for fluorospheres, and the standard deviation of the G0/G1 nuclei (SD) are recorded from each histogram.

Histogram peak shape was recorded as percent half peak coefficients of variation.

The haploid megagametophyte tissue of *Pinus radiata* was used to determine 1c and 2c channel number. As the haploid megagametophyte has 1c nuclei at the G0/G1 period, the channel number at where the G0/G1 peak occurred was designated as 1c channel number, the channel number at where the G2 peak occurred was designated as 2c channel number (Fig A-1) (Fig A: figures in the appendix).

## Preliminary Results




| | MIN | MAX | COUNT | PERCENT | MEAN | SD | %HPCV |
|---|---|---|---|---|---|---|---|
| 1 | 20 | 30 | 4770 | 47.0 | 24.6 | 1.5 | 4.68 |
| 2 | 42 | 52 | 4149 | 40.9 | 47.4 | 2.1 | 4.53 |

Fig A-1: Histogram of florescence intensity following PI staining of nuclei from the haploid megagametophyte tissue of *Pinus radiata* is shown in Fig A-1.

Peak 1: 1c nuclei at G0/G1 period, its mean channel number is 24.6.

Peak 2: 2c nuclei at G2 period, its mean channel number is 47.4.

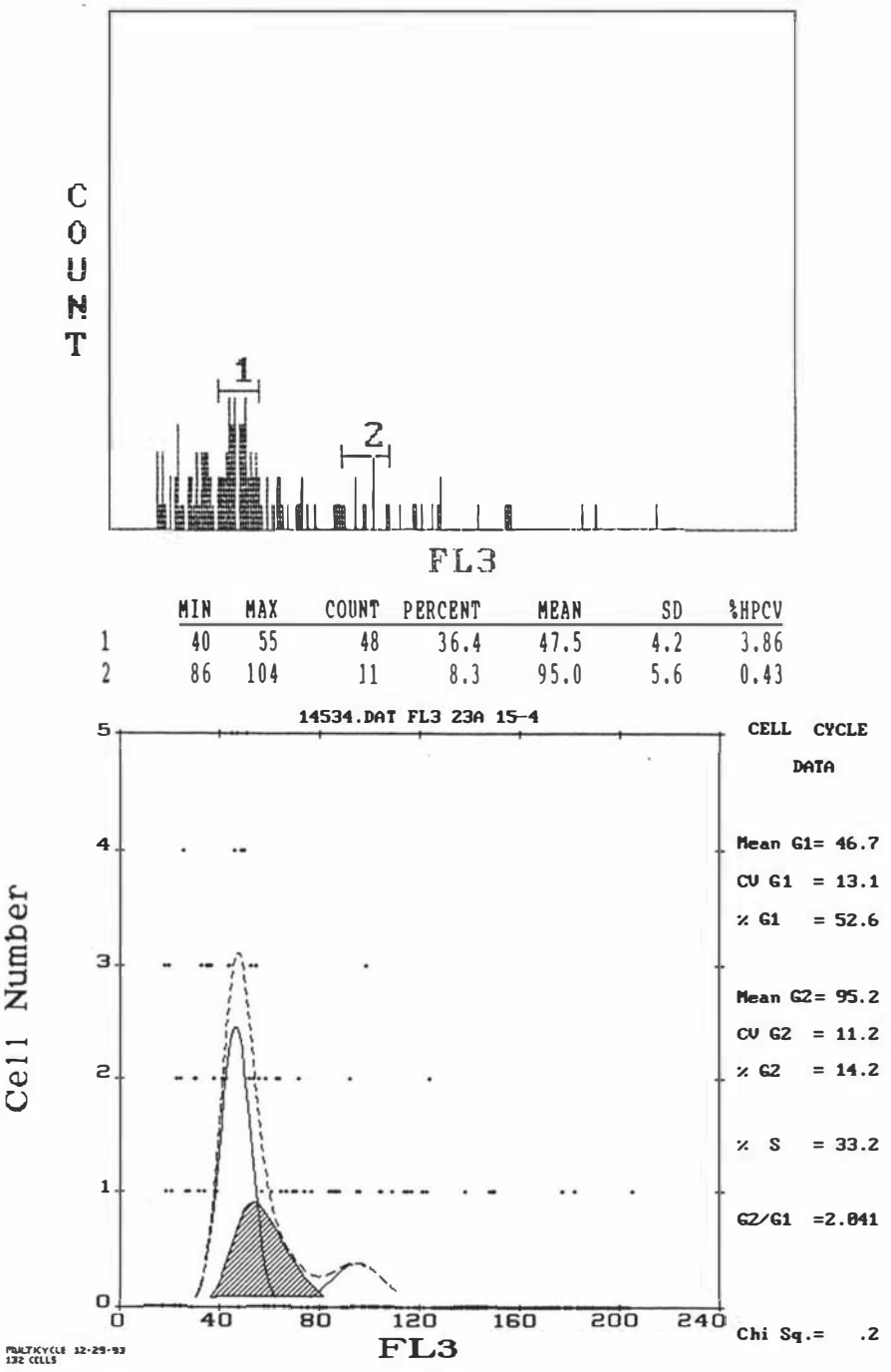
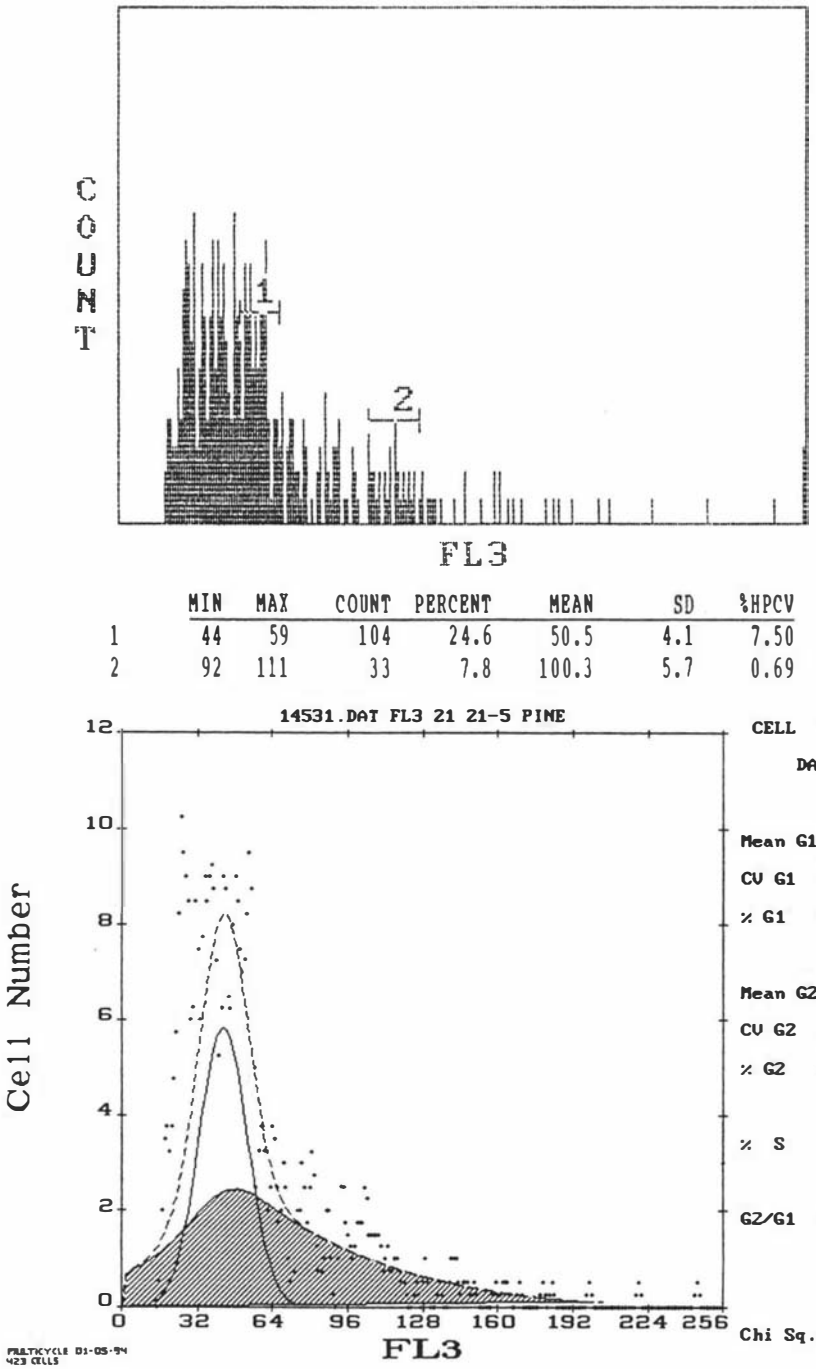



Fig A-2: Histogram of florescence intensity following PI staining of nuclei from sporogenous tissues of the male cone in *Pinus radiata* collected on 19/4/92.

Peak 1 : 2c nuclei at G0/G1 period, its mean channel number is 47.5,

Peak 2 : 4c nuclei at G2 period, its mean channel number is 95.0.

% S = 33.2

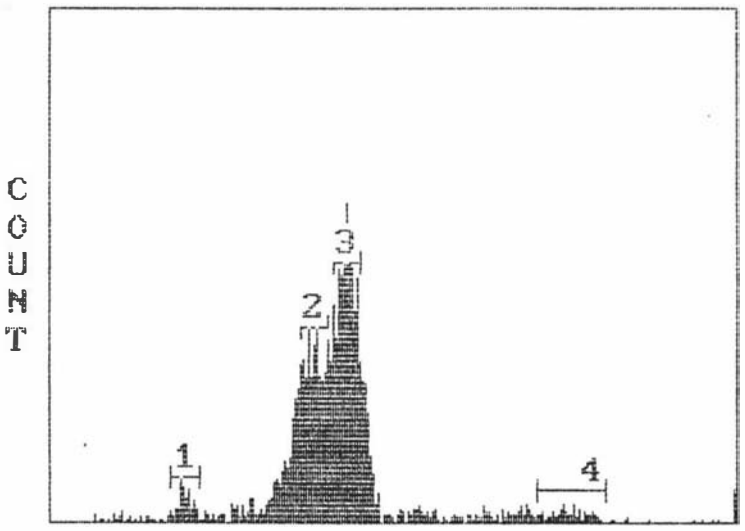



FigA-3 : Histogram of florescence intensity following PI staining of nuclei from sporogenous tissues of the male cone in *Pinus radiata* collected on 20/5/92.

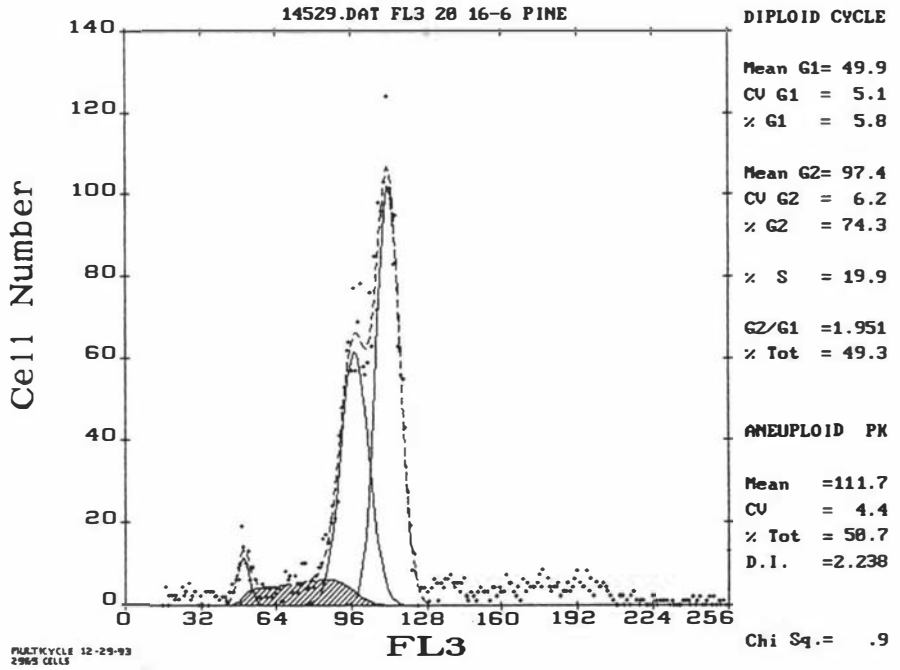
Peak 1 : 2c nuclei at G0/G1 period, its mean channel number is 50.5.

Peak 2 : 4c nuclei at G2 period, its mean channel number is 100.3.

% S = 60.3




| | MIN | MAX | COUNT | PERCENT | MEAN | SD | %HPCV |
|---|---|---|---|---|---|---|---|
| 1 | 44 | 55 | 99 | 3.3 | 49.5 | 2.7 | 3.74 |
| 2 | 93 | 103 | 713 | 24.0 | 98.0 | 3.2 | 12.2 |
| 3 | 105 | 115 | 1019 | 34.4 | 109.9 | 3.0 | 5.01 |
| 4 | 181 | 207 | 107 | 3.6 | 193.0 | 6.8 | 0.89 |



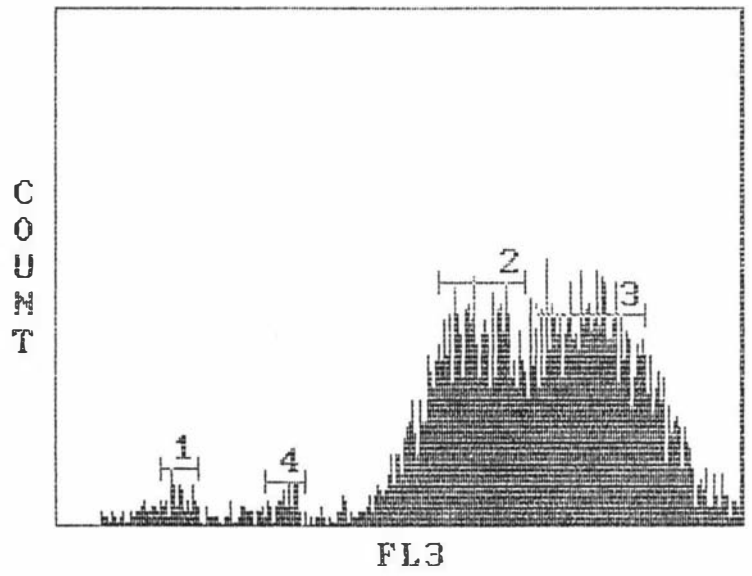

FigA-4 : Histogram of florescence intensity following PI staining of nuclei from sporogenous tissues of the male cone in *Pinus radiata* collected on 16/6/92.

Peak 1 : 2c nuclei at G0/G1 period, its mean channel number is 49.5.

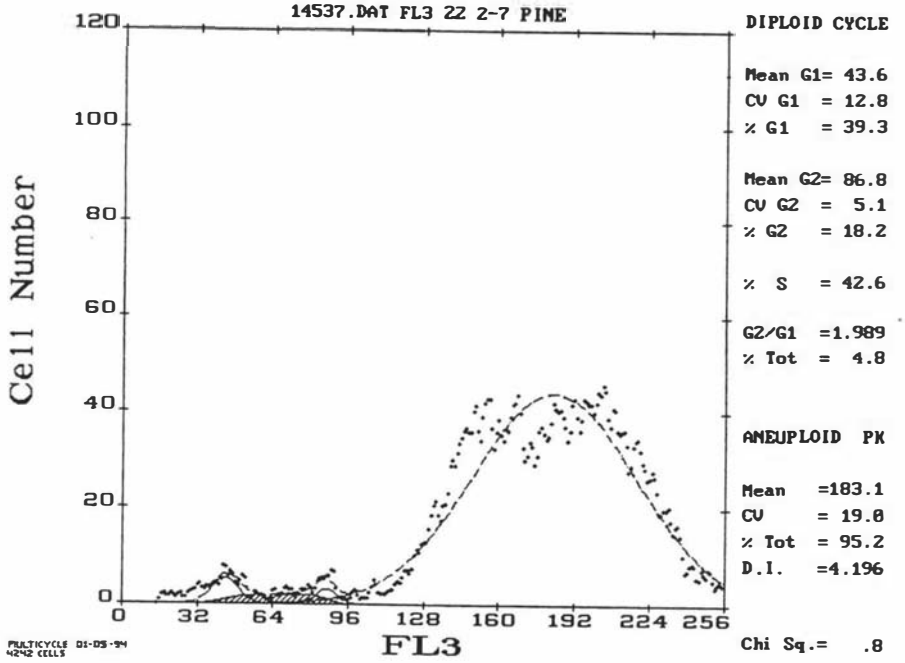
Peak 2, 3 : 4c nuclei at G2 period, their mean channel numbers are 98.0 and 109.9.

Peak 4 : 8c nuclei at G2 period, its mean channel number is 193.0.

% S = 19.9




| | MIN | MAX | COUNT | PERCENT | MEAN | SD | %HPCV |
|---|---|---|---|---|---|---|---|
| 1 | 38 | 52 | 82 | 1.9 | 44.9 | 4.1 | 2.26 |
| 2 | 142 | 174 | 1214 | 28.6 | 157.8 | 9.3 | 21.0 |
| 3 | 178 | 219 | 1606 | 37.9 | 198.0 | 11.8 | 9.03 |
| 4 | 77 | 92 | 71 | 1.7 | 84.7 | 4.0 | 0.99 |




FigA-5 : Histogram of florescence intensity following PI staining of nuclei from sporogenous tissues of the male cone in *Pinus radiata* collected on 2/7/92.

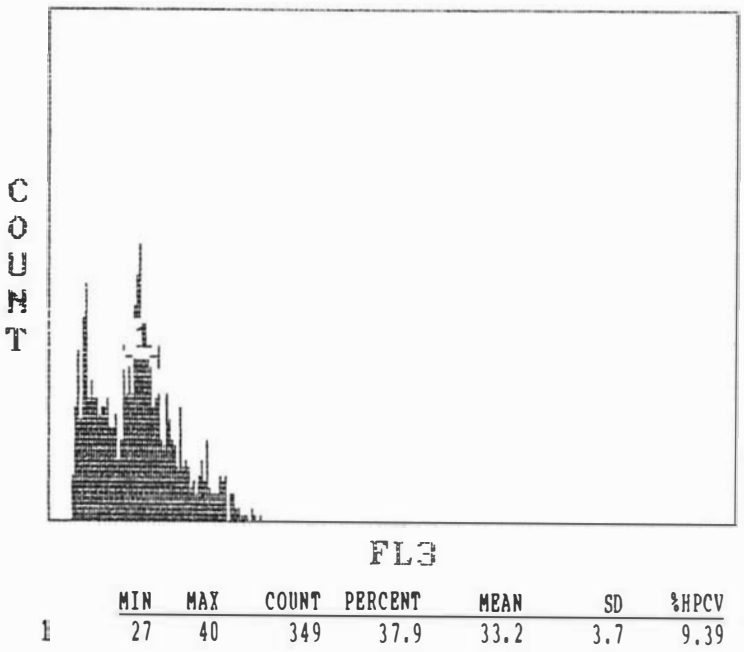
Peak 1 : 2c nuclei at G0/G1 period, its mean channel number is 44.9.

Peak 2 : 8c nuclei at G2 period, its mean channel number is 157.8.

Peak 3 : 8c nuclei at G2 period, its mean channel number is 198.0.

Peak 4 : 4c nuclei at G2 period, its mean channel number is 84.7.

% S = 42.6




| | MIN | MAX | COUNT | PERCENT | MEAN | SD | %HPCV |
|---|---|---|---|---|---|---|---|
| 1 | 27 | 40 | 349 | 37.9 | 33.2 | 3.7 | 9.39 |

FigA-6 : Histogram of florescence intensity following PI staining of nuclei from germinated pollen tubes of *Pinus radiata.*

Peak 1 : 1.5 c nuclei at G0/G1 period, its mean channel number is 33.2.

Table **A-1:** A summary of mean channel number of linear fluorescence in relation to the different DNA content (ploidy) of nuclei from tissues of *Pinus radiata* at different stages.

| Tissue analysed | 1c nuclei | 2c nuclei | 4c nuclei | 8c nuclei | 1.5c nuclei |
|---|---|---|---|---|---|
| Haploid megagametophyte tissue | 24.6 | 47.4 | | | |
| Male cone 19/4/92 | | 47.5 | 95.0 | | |
| Male cone 20/5/92 | | 50.5 | 100.3 | | |
| Male cone 16/6/92 | | 49.5 | 98.0, 109.9 | 193.0 | |
| Male cone 2/7/92 | | 44.9 | 84.7 | 157.8, 198.0 | |
| Pollen tube | | | | | 33.2 |

Table **A-2.** An interpretation of results of the flow cytometry study on changes of the nuclei ploidy during male cone development in *Pinus radiata.*

| Male cone 19/4/92 | Male cone 20/5/92 | Male cone 16/6/92 | Male cone | 2/7/92 | Pollen tube nuclei |
|---|---|---|---|---|---|
| pre prophase of meiosis | Prophase | Meiosis | Tetrads A population | Tetrads B population | |
| 2c nuclei | 2c nuclei, but approaching meiosis with a increased % S = 60.3. | 4c nuclei, two populations of cells are still not divided. They contain 2xDNA = 4c nuclei, but S - phase is very low. | 8c tetrad, 4x2c nuclei with expected 2c channel number values. | 8c tetrad, 4x2c nuclei with lower 2c channel number values because of chromatin condensation | 1.5c nuclei, according to the lower channel member value, but this could be a result of chromatin condensation. |
| | | | These 8c tetrad populations could be a result of either 3 or 4 nuclei's chromatin condensed together, each at 2c level. | | |

This flow cytometry study provided a preliminary result which suggested that the haploid 1c nuclei do not occur at the tetrad stage, they do not even appear in the germinated pollen tube in this species, *Pinus radiata.* This controversial result undoubtedly needs further detailed investigation, for example, the number of stained nuclei count needs to be increased to allow a more general and accurate channel number value, a consistent 1c and 2c nuclei control is also very important in this study. However, 2c tetrad and 2c pollen are not only seen in *Pinus*, differential chromatin condensation in Kiwifruit pollen with the vegetative nuclei at 2c and the generative nuclei at 1.5c have also been observed by Hopping *et al* (Personal communication).

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