A STUDY OF ANther gene function in
BRASSICA NAPUS USING AN ANTISENSE RNA
APPROACH.

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

by

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Dedicated to my wife Dilek
Acknowledgements

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1.1 Anther Development in Flowering Plants

Angiosperm plants produce flowers within which their reproductive development takes place. The flower carries the anthers and pistil in which the male and female gametophytes, respectively, are formed. The functions of the gametophytes are to produce the sperm cells and the egg cell, and promote their union at fertilization. The anther is a complex structure consisting of several cell-types, the most important of which include the microsporocytes and tapetal cells (Fig. 1.1). Microsporocytes or pollen mother cells are produced from the sporogenous tissue within the anther. The microsporocytes undergo meiosis to eventually form the pollen grains and, amongst other roles, the surrounding tapetal cells supply nutrients and structural components to the developing microspores (Nave and Sawhney, 1986; Mascarenhas, 1989). The
Figure 1.1
Cytological description of microgametogenesis in a typical angiosperm. Also shown are the relationship between bud length and anther developmental stage and the developmental windows covered by the Brassica napus cDNA libraries of Scott et al., 1991. Light shading represents callose, dark shading represents the exine of the pollen wall. (Abbreviations: A, archesporial cell; PP, primary parietal cell; PS, primary sporogenous cell; S, sporocyte; M, meiocyte; Me, meiosis; T, tetrad; MR, microspore release; MI, microspore interphase; DM, di-nucleate microspore; TP, Tri-nucleate pollen; "S", sporogenesis; "MD", microspore development.)
DEVELOPMENTAL STAGE

BUD LENGTH (mm)

1.0

LIBRARIES

"S"

"MD"

0.0

Microspore maturation

Microspore development

Sporogenesis
differentiation of the sporocytes is an early event in anther development and is characterized by the enlargement of the sporocytes after each mitosis, the synthesis of the callose wall, and meiosis (Scott et al., 1991b). Before meiosis, microsporocytes synthesize a complete wall of callose, a $\beta(1,3)$-glucan, between the cellulose cell wall and plasma membrane (Heslop-Harrison, 1968). Callose deposition continues throughout meiosis so that each of the products of meiosis, the tetrad of microspores, is also surrounded by callose. Pollen wall formation begins within the tetrad and all the principal features are elaborated before microspore release (Scott et al., 1991b). Thus, microspore development begins within the callose coat of the tetrad immediately following the completion of meiosis II. The enzyme activity callase, a mixture $\beta(1,3)$-glucanases, which is synthesized by the tapetal cells and secreted into the locule, breaks down the callose wall and releases the microspores into the locule (Eschrich, 1961; Heslop-Harrison and Dickinson, 1969; Mepham and Lane, 1969). However, in some species, including *Nicotiana tabacum* (tobacco) and *Brassica napus*, complete tetrad wall dissolution probably requires the participation of $\beta(1,4)$-glucanases (cellulases), since hydrolysis of the callose wall alone does not result in microspore release (Worrall et al., 1992). The timing of callase synthesis and activity is critical for microspore development. Early or late callase activity during microsporogenesis may result in male sterility (Izhar and Frankel, 1971). Therefore, perhaps early synchronisation takes place between tapetal cells and microspores to control the timing of callase production (Scott et al., 1991b). In addition to supplying tetrad wall degrading enzymes, the tapetum also supplies nutrients and structural components to the developing microspores (Nave and Sawhney, 1986; Sawhney and Nave, 1986). The significance of the tapetal cells in anther development is demonstrated by the fact that natural male sterility is often linked to tapetal malfunction (Kaul, 1988). Koltunow et al. (1990) and Mariani et al. (1990) reported that the tapetum plays a direct role in microspore development by showing that selective premature destruction of the tapetum causes male-sterility. Microspore development ends
when microspore mitosis produces a large vegetative cell and a smaller generative cell of the gametophyte proper. The generative cell nucleus eventually divides to produce the two sperm cells which fertilize the egg cell and endosperm nuclei (Mascarenhas, 1989). However, in some families, including the Brassicaceae, the mature pollen grain is trinucleate, since the second mitosis occurs during maturation. In most species examined, the tapetum degenerates during pollen maturation and is absent at anthesis, with most of the compounds released from the degenerating tapetum becoming adsorbed onto the surface of the pollen grain, forming the pollen coat (Heslop-Harrison, 1968).

The complex ontogeny described above is reflected in the complexity of the gene expression programme that is expressed during anther development. Kamalay and Goldberg (1980) reported that around 26,000 different transcripts are present during early anther development of tobacco and that about 11,000 of these are organ-specific. Approximately 20,000-24,000 different mRNAs are expressed in mature pollen of *Tradescantia paludosa* and *Zea mays*, of which 7200 are pollen-specific (Willing et al., 1984; 1988). They also found that approximately 60% of pollen-expressed transcripts are expressed in the vegetative shoot as well and that pollen-expressed genes appear to produce more abundant mRNA levels than their counterparts in shoots. In *Tradescantia* pollen, 75% of total mRNA derives from abundantly expressed genes, whilst only 35% of shoot mRNA consists of highly expressed transcripts.

1.2 Cloning of Genes Expressed During Anther Development

The construction and differential screening of cDNA libraries derived from anther mRNA offered a straightforward means of cloning anther-specific genes (Table 1). Several laboratories have constructed cDNA libraries to poly(A) RNA from anthers and pollen grains. The temporal and spatial expression of the transcripts identified by differential screening reflect the origin of the mRNA used in library construction. Scott *et al.* (1991b) found a strong relationship between anther length and the stage of microgametogenesis in *B. napus*. So they were
able to construct defined cDNA libraries; namely, sporogenesis and microspore
development. Thus the temporal specificity of the cDNA clones isolated from the
two libraries paralleled the temporal specificities of the corresponding library.

In general the most powerful means of cloning cell-specific cDNAs is to
isolate the appropriate cell type for construction of libraries. However, this
approach has limited application to anther development because of the difficulty
of purifying important cell types such as tapetal cells and sporocytes. On the
other hand, the ease of collection of mature pollen grains enabled cDNA
libraries to be constructed which have produced a variety of pollen-specific
clones. Hanson et al. (1989) characterized the Zmc13 transcript from maize and
showed its expression during pollen germination and pollen tube growth as well
as pollen maturation. McCormick et al. (1987) isolated the LAT52 cDNA clone
from a tomato mature-anther library and showed that this possessed sequence
similarity to Zmc13 at the amino acid level. Ursin et al. (1989) showed that the
LAT52 message is located in mature pollen grains and in the anther wall. The
LAT52 gene of tomato was isolated and it was demonstrated that the LAT52
promoter was active with the same temporal and spatial expression patterns in a
number of transgenic plants (Twell et al., 1989; 1990). The Zm13 5' flanking
region also showed similar results (Hamilton et al., 1989; Guerrero et al., 1990)
suggesting that the mechanisms which regulate pollen-specific gene expression
are conserved across wide evolutionary distances. In terms of the anther, whole
anther libraries remain the most numerous and useful. Koltunow et al. (1990)
investigated the spatial expression patterns of some anther-specific transcripts
using cDNAs isolated from an immature anther library of tobacco. They found
that three cDNAs (TA26, TA29 and TA32) out of five were localised to the
tapetum. Smith et al. (1990) reported three tapetum-specific genes from tomato.
Scott et al. (1991a) constructed two anther cDNA libraries, one from anthers of
1.2-1.8 mm long buds (sporogenesis library) and one from anthers of 1.8-4.0
mm long buds (microspore development library) of B. napus. Three cDNAs
Table 1.1 Anther specific/expressed cDNA clones isolated from various plant species.

<table>
<thead>
<tr>
<th>cDNA clone</th>
<th>Species</th>
<th>Specificity</th>
<th>Identity</th>
<th>References</th>
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<tr>
<td>Zmc13</td>
<td><em>Zea mays</em></td>
<td>Pollen</td>
<td>Unknown</td>
<td>Hanson et al. 1989</td>
</tr>
<tr>
<td>LAT52</td>
<td><em>Lycopersicon esculentum</em></td>
<td>Pollen, anther wall</td>
<td>Unknown</td>
<td>Twell et al. 1989</td>
</tr>
<tr>
<td>LAT56, LAT59</td>
<td><em>L. esculentum</em></td>
<td>Pollen, root</td>
<td>Pectate lyase of <em>Erwinia</em></td>
<td>Wing et al. 1989</td>
</tr>
<tr>
<td>TA13, TA29</td>
<td><em>Nicotiana tabacum</em></td>
<td>Tapetum</td>
<td>Glycine-rich protein</td>
<td>Koltunow et al. 1990</td>
</tr>
<tr>
<td>TA32, TA36</td>
<td><em>N. tabacum</em></td>
<td>Tapetum</td>
<td>Lipid transfer protein</td>
<td>Koltunow et al. 1990</td>
</tr>
<tr>
<td>TA20</td>
<td><em>N. tabacum</em></td>
<td>Con, St, pistil</td>
<td>Unknown</td>
<td>Koltunow et al. 1990</td>
</tr>
<tr>
<td>TA25</td>
<td><em>N. tabacum</em></td>
<td>Nd</td>
<td>Unknown</td>
<td>Koltunow et al. 1990</td>
</tr>
<tr>
<td>TA26</td>
<td><em>N. tabacum</em></td>
<td>Tapetum</td>
<td>Unknown</td>
<td>Koltunow et al. 1990</td>
</tr>
<tr>
<td>TA56</td>
<td><em>N. tabacum</em></td>
<td>Con, St</td>
<td>Thiol peptidase</td>
<td>Koltunow et al. 1990</td>
</tr>
<tr>
<td>P2</td>
<td><em>Oenothera organensis</em></td>
<td>Pollen</td>
<td>Polygalacturonase</td>
<td>Brown and Crouch 1990</td>
</tr>
<tr>
<td>108, 127a, 92b</td>
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<td>Unknown</td>
<td>Smith et al. 1990</td>
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<tr>
<td>Bcp1</td>
<td><em>B. campestris</em></td>
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<td>Unknown</td>
<td>Theerakulpisut et al. 1991</td>
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<td>Bp19</td>
<td><em>B. napus</em></td>
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<td>Pectin esterase</td>
<td>Albani et al. 1991</td>
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<td>tap2</td>
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<td>Tapetum</td>
<td>Unknown</td>
<td>Nacken et al. 1991</td>
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<tr>
<td>BA112, BA158</td>
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<td>Tapetum</td>
<td>Unknown</td>
<td>Shen and Hsu 1992</td>
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<tr>
<td>BA42</td>
<td><em>B. napus</em></td>
<td>Ta, Vb</td>
<td>Chalcone synthase</td>
<td>Shen and Hsu 1992</td>
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<tr>
<td>BA54, BA73</td>
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<td>Pollen, Nd</td>
<td>Unknown</td>
<td>Shen and Hsu 1992</td>
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<tr>
<td>I3</td>
<td><em>B. napus</em></td>
<td>Pollen</td>
<td>Oleosin</td>
<td>Roberts et al. 1993</td>
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</table>

Con, connective; St, stomium; Ta, tapetum; Vb, vascular bundle; Nd, not determined
(A3, A8 and A9) isolated from the sporogenesis library were shown by in situ hybridization to be tapetum-specific. Also, the temporal expression patterns of the A3 and A9 transcripts were very similar. These reports revealed that tapetal messages dominate the mRNA population of the young anther and therefore they appeared to be the high abundance class of transcripts. Thus, the tapetum produces a number of highly expressed mRNAs that are consequently easily identified by differential screening. However, in contrast to the tapetum the sporogenous cells appears not to synthesise a class of highly abundant cell-specific mRNAs (Scott et al., 1991b). This may reflect the reduction in the amount of mRNA and rRNA during meiosis that occurs in this cell type (Porter et al., 1984).

The pattern of anther gene expression changes by the end of meiosis when the young microspores appear. Although the tapetum continues to produce a range of highly abundant and cell-specific transcripts, the post-meiotic mRNA pool contains abundant transcripts derived from the microspores and from several different cell types within the anther wall (Scott et al., 1991b). It was suggested that there are two classes of genes with respect to the timing of their activity during pollen development (Stinson et al., 1987; Mascarenhas, 1989). The first set of genes (group I) are activated after microspore mitosis, and the mRNAs increase in concentration up to maturity. The second group (group II), which includes microspore expressed genes like actin and alcohol dehydrogenase, are active soon after meiosis is completed and the mRNAs reach their maximum accumulation around the time of microspore mitosis, then reduce substantially by anthesis (Stinson and Mascarenhas, 1985; Stinson et al., 1987). These are expressed in the microspores following release from the tetrad, and disappear before the formation of the mature pollen grain. Also, recently several microspore-specific cDNA clones showing group II - type expression have been isolated from B. napus (Albani et al., 1990; 1991 and Scott et al., 1991a). With the exception of the Bp19 gene of B. napus, which is reported to have pectin esterase activity (Albani et al., 1990), and the I3 cDNA
clone of *B. napus* which encodes an anther-specific oleosin (Roberts *et al.*, 1993b), the function of the remaining early microspore specific group II genes is unknown. Of the later expressed anther- and pollen-specific genes, LAT56 and LAT59 from tomato encode proteins with similarities to pectate lyases of *Erwinia* and it is suggested that they might relate to a requirement for pectin degradation during pollen tube growth (Wing *et al.*, 1989). Similarly the P2 gene product from *Oenothera organensis* which is present in germinating pollen tubes, has an identity with tomato polygalacturonase (Brown and Crouch, 1990). Koltunow *et al.* (1990) reported that the TA56 cDNA clone from tobacco encodes a thiol peptidase, and is localized in the anther connective and stomium, maximally at the time of anthesis. Therefore, this gene appears to be involved in the degradation of anther wall tissues leading to dehiscence. However, TA20, TA25 and TA26 cDNA clones failed to show relatedness to any known mRNA or protein. To date, 13 cDNA clones out of a total of 27 anther-specific or anther-expressed cDNA clones have shown significant amino acid sequence similarity to known proteins (Table 1.2). A total of 14 therefore, have failed to score significant matches with data-base sequences. There are many other cDNAs or cognate mRNAs isolated from various plant organ types or induced by various environmental factors that cannot presently be assigned a function.

Thus, a significant challenge in biology is to assign function to a large number of genes that have been identified by molecular biological techniques. When data-base searches fail to find a significant match for a newly isolated sequence, there are several directions that could be considered in order to elucidate function: 1) make an antibody to determine cellular localisation of the protein of interest, 2) to obtain the corresponding gene and link the promoter to reporter genes (e.g. *gus* (Jefferson *et al.*, 1987) or barnase (Mariani *et al.*, 1990)) to determine the exact tissue specificity and temporal regulation, 3) to use reverse genetics to create a mutant phenotype. One or more of these procedures is usually followed even when a match is made, since sequence similarity alone does not prove that the gene product fulfils that function. For
example, Albani et al. (1992) demonstrated that the genomic clone Bp10 corresponded to a small pollen-specific gene family of *B. napus* showing sequence similarity to ascorbate oxidase (AAO). However, when Bp10 protein was over-expressed in *E. coli* and tested for AAO activity it failed to show the predicted enzymatic function.

The first two approaches listed above can provide valuable clues but alone are unlikely to reveal the function of the gene. In contrast, reverse genetics via antisense RNA expression has been successful in assigning function to various new sequences by creating diagnostic mutant phenotypes. One of the principal aims of the work presented in this thesis was to evaluate an antisense approach in the characterisation of cDNAs cloned from an anther-specific cDNA library.

1.3 Reverse Genetics

Unlike classical genetics, "reverse genetics" starts with a cloned gene and then attempts to elucidate an exact role or identity. In reverse genetic methods, wild type genetic functions are disrupted, or replaced in a targeted manner, by transformation with new genetic elements based on the nucleotide sequence of the gene of interest. The most important advantage of the reverse genetic approach is that where an interesting mutant is successfully produced, the gene (or probe for the gene) is of course available immediately. In forward genetics, following the identification of the mutant, a great deal of effort is required in order to then clone the gene. On the other hand, making mutants by classical means allows all "possible" mutants to be isolated (in theory). One can then decide what is interesting before embarking on gene cloning. The question is then, is it more efficient to create many antisense mutants until the required gene or class of genes is identified, or to create mutant plants with the relevant characteristics by, for example T-DNA insertional inactivation, and then clone the gene? The use of sense/antisense RNA constructs represents only one of the reverse genetic methods, but it is the most practicable strategy for gene identification by reverse genetics.
Antisense RNA technology is based on blocking the flow of information from DNA via RNA to protein by the introduction of an RNA strand complementary to the sequence of the target mRNA (Mol et al., 1990). Regulation of gene expression by antisense RNA was first discovered as a naturally-occurring phenomenon in bacteria (Simons, 1988). Izant and Weintraub (1984) first reported the use of antisense RNA constructs in eukaryotic cells. The antisense approach in eukaryotes is now well integrated in the field of molecular and applied genetics. However, the mechanism of antisense inhibition is not understood clearly but is presumed to involve duplex formation (Mol et al., 1990). There are some theories on the mechanism of antisense inhibition: 1) double-stranded RNA is selectively degraded by RNase, 2) antisense RNA blocks the poly (A) signal (this may inhibit transportation of double-stranded RNA from nucleus to cytoplasm), 3) antisense RNA blocks the capping reaction and inhibits transcription, 4) antisense RNA covers the AUG site and inhibits translation, 5) antisense RNA covers the exon-intron junction to block the splicing reaction. Apparently, the presence of the target gene in the nucleus is unaffected by antisense RNA in transgenic plants though mRNA is downregulated. This shows that the antisense effect is post-transcriptional. Ecker and Davis (1986) first reported antisense inhibition of gene expression in plants. They achieved effective transient inhibition of chloramphenicol acetyl transferase (CAT) activity in carrot cells. Nopaline synthase (NOS) activity was inhibited by double transformation of tobacco plants with NOS and antisense NOS genes (Rothstein et al., 1987; Sandler et al., 1988). Subsequently, other bacterial genes such as bar (Cornelissen and Vanderwiele, 1989) and gus (Robert et al., 1989) have been downregulated by the introduction of antisense RNA to transformed tobacco plants. The reduction in GUS activity correlated with the levels of GUS enzyme and the steady state GUS mRNA observed in antisense transformed plants. The gene encoding chalcone synthase (CHS), a key enzyme in flavonoid biosynthesis pathway, was the first plant gene successfully downregulated by antisense RNA (Van der Krol et al., 1988).
Flower pigmentation was altered in antisense transgenic petunia and tobacco plants due to a reduction in the level of both the CHS message and enzyme activity, and these phenotypes were stably inherited. Antisense CHS genes encoding half-length or quarter-length RNA complementary to the 3' half of the CHS mRNA also successfully altered flower pigmentation (Van der Krol et al., 1990). These experiments failed to demonstrate that the mechanism of antisense is via the formation of double-stranded RNA since neither duplex RNA nor free antisense RNA could be detected in transgenic plants. Smith et al. (1988, 1990) and Sheehy et al. (1988) achieved a dramatic reduction of polygalacturonase (PG) mRNA and PG protein levels after introduction of an antisense PG gene in tomato. Polygalacturonase (PG) plays an important role in fruit ripening and the objective was to increase shelf life of tomato using antisense technology. Rodermel et al. (1988) reported downregulation of ribulose bisphosphate carboxylase (RUBISCO) small subunit mRNA and protein using antisense RNA, and that these changes were also accompanied by reductions in RUBISCO-large subunit protein but not RUBISCO-large subunit mRNA amounts. RUBISCO, which is a chloroplast-located enzyme, is composed of small and large subunits encoded by a nuclear multigene family and a single chloroplast gene, respectively. Amylose-free potatoes were obtained by introduction of an antisense granule-bound starch synthase gene (Visser et al., 1991). Antisense technology has also been successfully used to understand the function of proteins in transgenic plants. The functional identity of several cDNA that had failed to score significant sequence data-base matches has been achieved by antisense RNA expression in transgenic plants. These include cDNAs which encode the ethylene forming enzyme ACC-oxidase and phytoene synthase, an enzyme of the carotenoid biosynthetic pathway (Hamilton et al., 1990; Bird et al., 1991).

Antisense RNA techniques have also been successfully employed to interfere with normal anther function. For example, the inhibition of flavonoid biosynthesis in petunia anthers resulted in male sterility (Van der Meer et al.,
1992). Insertion of anther box into the CaMV 35S promoter directed antisense CHS gene expression in tapetum cells and downregulated pigmentation in anthers of transgenic plants. This led to a block in pollen development that resulted in male sterility. In petunia, CHS comprises a multigene family (Koes et al., 1987) and thus the above report demonstrated that antisense RNAs have the ability to inhibit expression of gene families. Knutzon et al. (1992) modified Brassica seed oil compositions by antisense expression of a stearoyl-acyl carrier protein desaturase gene. This result showed that antisense technology can be used successfully to engineer seed oil compositions. Previous reports showed that about 40% of transformants gave a phenotype which revealed that antisense is not always efficient. Also, the downregulation of plant mRNAs without obvious phenotypic effect has been reported. Neuhaus et al. (1992) downregulated the expression of a 6(1,3) glucanase in tobacco and Hall et al. (1993) the expression of a pectin esterase (PE) gene in tomato, both without phenotypic effects.

There are several prerequisites for the application of antisense RNA technology. Most critical are a certain level of nucleotide conservation between the antisense RNA and target mRNA, and the expression of antisense gene in the same cell as target mRNA. Unfortunately, the exact extent of sequence similarity required between target and antisense RNAs has proved difficult to quantify. Antisense CHS genes encoding half-length or quarter-length RNA complementary to the 3' half of CHS mRNA altered flower pigmentation, whilst a gene encoding RNA complementary to the 5' half of CHS mRNA did not show phenotypic effects in transgenic petunia plants (Van der Krol et al., 1990). Cannon et al. (1990) downregulated leaf-specific GUS gene expression in transgenic tobacco plants using an antisense RNA with a 41-base homology spanning the translation start codon of the gene. These reports demonstrated that subgenomic parts of the gene could be as effective as whole gene to inactivate gene expression.

The CaMV 35S promoter has been widely used to direct the transcription
of the antisense genes in transgenic plants. Flower pigmentation was inhibited by expressing the antisense CHS gene driven by the CaMV 35S promoter in transgenic petunia plants (Van der Krol et al., 1988), polygalacturonase (PG) gene expression was downregulated with CaMV 35S promoter-antisense PG gene expression in transgenic tomato plants (Smith et al., 1988). Also, RUBISCO enzyme level in transgenic tobacco plants (Rodermel et al., 1988), synthesis of ethylene in transgenic tomato plants (Hamilton et al., 1990) and tonoplast H+ ATPase enzyme level in carrot (Gogarten et al., 1992) were inhibited by introducing antisense genes under the control of the CaMV 35S promoter. However, CaMV 35S-GUS expression in anthers showed that the CaMV 35S promoter did not express in the tapetum and microspores (Plegt and Bino, 1989; Van der Meer et al., 1992). Further evidence was provided by the fact that expression of a modified glucanase gene in the anther caused dissolution of the sporocyte callose when driven by the tapetum-specific promoters, A3 and A9, but not the 35S promoter (Worrall et al., 1992). Van der Meer et al. (1992) also reported that antisense CHS gene driven by the CaMV 35S promoter was not effective in inhibiting pigmentation in anthers. However, modification of the 35S promoter by the insertion of an "anther box" - a short promoter sequence found in other anther-expressed genes - resulted in the downregulation of pigmentation in anthers of transgenic petunia plants.

The principal aim of the work presented in this thesis was to apply an antisense RNA approach to determine the function of two anther-specific cDNA clones, A1 and A9. Both were isolated by differential screening from a cDNA library constructed from B. napus anthers dissected from 1.2-1.8 mm long buds (sporogenesis library) (Scott et al., 1991a). A1 transcript is present during the tetrad and microspore-release stages of microsporogenesis, although the exact cellular location was unknown. The predicted amino acid sequence of A1 showed similarity to both chalcone synthase and stilbene synthase enzymes. In situ hybridization indicated that the A9 cDNA clone represents a tapetum-specific transcript (Scott et al., 1991a; Paul et al., 1992). The close phylogenetic
relation between *B. napus* and *Arabidopsis thaliana* was exploited to facilitate the isolation of the A9 gene from *A. thaliana*. This confirmed that generally *B. napus* cDNAs have well conserved homologous in *A. thaliana*. A9 promoter-reporter gene fusions were then characterised in transgenic plants and provided confirmation of the tapetum-specificity of the A9 gene. Although, the deduced A9 protein sequence exhibited a pattern of cysteine residues that is present in seed storage proteins and several protease and α-amylase inhibitors (Paul et al., 1992), the extent of homology shared with these proteins was very low and therefore the function of the A9 protein was uncertain.

More specifically then, the aims of the project were to determine whether A1 encoded chalcone or stilbene synthase, and to obtain further clues about the biological function of the A9 gene product. The realisation of these aims by an antisense RNA approach would require the expression of the antisense RNA in the correct anther cell type. The *A. thaliana* A9 promoter had been cloned previously (Paul et al., 1992) and was therefore available to drive appropriate A9 antisense RNA expression. In contrast, the cellular localisation of the A1 message was unknown at the outset and therefore an early goal was to determine the cell specificity of this transcript. Authentic A1 and A9 messages are specifically expressed in *Brassica* anthers. Therefore, antisense RNA inhibition required gene transfer to *Brassica* or perhaps *Arabidopsis*. This was problematical since gene transfer to these species was under-developed when the work reported here began.

1.4 Gene transfer to *Brassica napus* and *Arabidopsis thaliana*

Although in some circumstances, transient expression of antisense mRNA is useful, in most instances antisense RNA constructs should be stably integrated into higher plants to investigate the role of the target mRNA using an appropriate transformation method. Stable transformation is certainly required for antisense experiments in fully differentiated organs such as the anther. To achieve this, *Agrobacterium tumefaciens*-mediated transformation is the most convenient
method. Although tobacco transformation is facile, the present work required gene transfer to *Brassica* and *Arabidopsis*, since the target mRNAs, A9 and A1, either do not occur in tobacco or are too widely diverged at the level of nucleotide sequence to cross-hybridize with their tobacco counterparts.

*Agrobacterium tumefaciens* is a soil bacterium which induces crown galls at wound sides on dicotyledons (De Cleene and De Lay, 1976). Tumour tissues synthesize novel amino acid and sugar derivatives which are called opines. The type of opine (e.g. nopaline, octopine) is depend on the strain of *Agrobacterium* that initiated tumour formation. All virulent strains of *A. tumefaciens* carry an extrachromosomal element which is called the Ti-plasmid (tumour inducing) because of its role in tumour induction (Van Larebeke *et al.*, 1975). During the course of infection, part of the Ti-plasmid is introduced into the plant genome, this is the T-(transferred) DNA which is defined by flanking 25-base pair directly repeated sequences (Zambryski *et al.*, 1983). Genetic analysis has shown that the T-DNA and vir (virulence) region are associated with tumour formation. One of the important step during bacterial infection via wounded tissues is the co-ordinate activation of the virulence system (Stachel and Zambryski, 1986).

Stachel *et al.* (1985) identified these plant activators (inducers) from tobacco as being the phenolic compounds acetosyringone and α-hydroxyacetosyringone. The available evidence suggests that the T-DNA does not integrate at specific positions in the nuclear genome. Seven T-DNA inserts were mapped on five different chromosomes of tomato (Chyi *et al.*, 1986). Tumour formation is a complex process which involves a number of steps including recognition of plant target cells by *Agrobacterium*, attachment of the bacterium to the plant cells, T-DNA transfer, T-DNA integration into the plant genome, T-DNA expression, and symptom expression by the transformed plant cells (Hooykaas and Schilperoort, 1992). Two types of vector system have been developed for plant transformation: 1) *cis* systems in which new genes are introduced into the T-DNA via homologous recombination (Zambryski *et al.*, 1983), and 2) binary systems in which new genes are cloned into small plasmids containing an
artificial T-DNA, which are subsequently introduced into an Agrobacterium strain harbouring a modified (T-DNA minus) Ti plasmid (Bevan, 1984). These vector systems have been developed by the addition of selection markers; for instance neomycin phosphotransferase (NPTII) gene for kanamycin resistance (Bevan et al., 1983) and reporter genes such as β-glucuronidase (GUS) (Jefferson et al., 1987). The Agrobacterium vector system is being used extensively to transform crop plants as well as for the study of gene function in plants.

B. napus (oilseed rape) and A. thaliana have been transformed using various explants by Agrobacterium vectors. Both species are natural hosts for Agrobacterium. Ooms et al. (1985) reported production of hairy roots and tumour formations from oilseed rape respectively by infection of A. rhizogenes and A. tumefaciens. Transgenic B. napus plants were obtained by in vitro inoculation of excised stem segments with A. rhizogenes (Guerche et al., 1987). Fry et al. (1987) obtained transformed B. napus plants using stem segments with an A. tumefaciens strain containing a disarmed Ti-plasmid. Agrobacterium-mediated transformation of thin cell layer explants yielded transgenic B. napus plants (Charest et al., 1988). Hypocotyl explants have been used to transform oilseed rape by A. tumefaciens (Radke et al., 1988). B. napus was readily infected by A. tumefaciens nopaline strains but not octopine strains (Holbrook and Miki, 1985; Charest et al., 1989). Moloney et al. (1989) reported transformation of B. napus cotyledonary explants using A. tumefaciens vectors. However, the reported results have been difficult to reproduce (Khehra and Mathias, 1992). Although Agrobacterium-mediated transformation of B. napus has been achieved using various explant types, the frequency of transformation still remains very low and needs to be improved. This is problematical because numerous reports have shown that only a proportion of transgenic plants carrying an antisense gene exhibit any phenotypic effects (Van der Krol et al., 1988, 1990; Bird et al., 1991; Van der Meer et al., 1992). Therefore a high frequency of transformation is desirable in order to examine the phenotypic effect in a large number of transgenic plants. It was suggested that phenotypic
variations between independent transformants were probably due to position effects (Van der Krol et al., 1990; Van der Meer et al., 1992). Therefore, the site of integration of antisense genes into the plant nuclear genome could alter patterns of expression of transferred genes.

*A.* *thaliana* has found utility in studies on basic physiology and biochemistry as well as in plant molecular genetic manipulations and developmental biology research due to its small genome, low chromosome number, short regeneration time, availability of many mutants and genetic maps, sexual self-compatibility and prolific seed production. More extensive use of *A.* *thaliana* has been hampered because of difficulties in efficient and rapid regeneration and transformation procedures. Transgenic *A.* *thaliana* plants were obtained from leaf explants (Lloyd et al., 1986) and from stem explants (An et al., 1986) via *Agrobacterium*-mediated transformation. Feldmann and Marks (1987) achieved transformation of germinating seeds of *A. thaliana* using *A. tumefaciens*. However, this method has proved difficult to repeat and is therefore not used routinely. Efficient *Agrobacterium*-mediated transformation of *A. thaliana* was reported using root explants (Valvekens et al., 1988). Transformed *A. thaliana* plants were also recovered using direct gene transfer to protoplasts (Damm et al., 1989). Despite these reports, the frequency of transformation remains low and the production of transformed shoots is generally protracted.

1.5 Genetic Engineering for Male Sterility

Male sterility is one of the most desirable systems for the production of hybrid plants for the plant breeder and seed producer. Hybrid seed production on a large scale is challenging since many crops have both male and female organs on the same plant, either within a single flower (e.g. oilseed rape, tomato) or in separate flowers (e.g. *Zea mays*). Therefore, mechanical or hand emasculation of the female line is required to prevent self-pollination for hybrid production, making seed production expensive. *B. napus* is being studied to improve oilseed production and agronomic characteristics by the plant breeders. Also,
seed companies have concentrated on hybrid seed production from oilseed rape due to its commercial importance. Therefore, introducing a versatile and durable male-sterility system for oilseed rape would be commercially attractive. The idea is a simple one in which an anther-specific promoter is used to drive the expression of a ‘disrupter’ gene that is capable of destroying some vital component of the anther thereby causing pollen production to fail (Scott et al., 1991b). Since natural male sterility is often linked to tapetal malfunction (Kaul, 1988), researchers concentrated on destruction of the tapetum to create male sterile plants using tapetum-specific promoters. When the tapetum-specific promoter TA29 used to drive expression of the Bacillus amyloliquifaciens RNase, barnase, in transgenic plants this resulted in the destruction of the tapetum and, as expected, in male sterility due to a failure to produce pollen grains (Mariani et al., 1990). Importantly, female fertility was not affected by this genetic system. Antisense inhibition of flavonoid biosynthesis in Petunia anthers also resulted in male sterility (Van der Meer et al., 1992).

Genetically engineered male sterility has some advantages over natural cytoplasmic male sterility (CMS) (Scott et al., 1991b). First, since the sterility gene is introduced into the genome by artificial means, the disruption of the genotype of the new male sterile is less than caused by the sexual transfer of new cytoplasts. Secondly, since sterility is not linked to a particular cytoplasm, the risk of linkage to disease susceptibility is also reduced. In corn, for nearly two decades CMS-T (cytoplasmic male sterility-T) was used in hybrid production in order to avoid hand or mechanical emasculation. However, a fungal disease known as Southern corn leaf blight, which is caused by Bipolaris maydis race T, occurred in epidemic proportions and decimated the corn crop in USA. It was apparent that CMS-T was directly related to the blight disease because male sterility and disease susceptibility are associated with a mitochondrial gene (Levings, 1990). Thirdly, it is possible to use a single sterility gene in widely diverged species since there is remarkable conservation of the elements controlling expression specificity. The restoration of male fertility in the F1 hybrid
is very important if the harvested product is seed and therefore the use of male sterility requires a fertility restorer system. The problem arises because engineered sterility is dominant.

In natural systems, fertility is restored by nuclear genes carried by the pollen-parent of the final cross. However, male fertility was successfully restored to genetically engineered male sterile oilseed rape plants (Mariani et al., 1992). Male sterile plants that expressed the barnase gene in the anther tapetal cell layer were crossed with male fertile plants that were transformed with a chimaeric tapetum-specific ribonuclease-inhibitor gene, barstar. F1 progeny expressing both genes are restored to male fertility by the suppression of cytotoxic ribonuclease activity in the anther by the formation of cell-specific RNase/RNase inhibitor complexes.

At the time of initiating the work described in this thesis it was hoped that an antisense strategy could be used to create male sterility. However, for such an approach it must be confirmed that the target mRNA is essential for male fertility. This approach was started before the barnase/barstar system was published. Moreover, there are still doubts about this system: what are the effects of using an aggressive ribonuclease under field condition; and will people object to its use? So perhaps an antisense/ribozyme approach would still have some merit.

1.6 General Aims

The thesis aim was use sense antisense RNA to alter/inactivate nuclear-encoded genes in order to create mutants and thus identify the function of a previously cloned gene. It was intended to work on flower development of B. napus (oilseed rape) with the aim of using sense/antisense RNA constructs to create mutant plants in order to identify cDNA clones of genes having an essential function during anther development. At the outset it was envisaged that a large number of cDNAs could be subjected to reverse genetics. Therefore, an important part of the project was to improve Agrobacterium-mediated
transformation of *B. napus* and *A. thaliana*, since at the time, this appeared the limiting factor. Why was oilseed rape utilised? Firstly, *B. napus* has great economic value world-wide. Secondly, under suitable conditions, flowering occurs in less than two months, and it has an indeterminate flowering habit, thus making it possible to collect all stages of bud development from a single plant at the same time for cytological and molecular analysis. *A. thaliana* was used as a model plant because of its close phylogenetic relationship with *B. napus*. It possesses a short generation time, low chromosome number and small size.
CHAPTER 2
MATERIALS AND METHODS

2.1 Bacterial Culture

2.1.1 Bacterial Strains

The following wild-type, oncogenic Agrobacterium strains obtained from Dr. John Draper were used to determine the most appropriate vir system for transformation of Brassica napus.

<table>
<thead>
<tr>
<th>Agrobacterium Strains (Wild-type)</th>
<th>Octopine/Nopaline</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACH5</td>
<td>Octopine-strain</td>
</tr>
<tr>
<td>C58</td>
<td>Nopaline-strain</td>
</tr>
<tr>
<td>A6</td>
<td>Octopine-strain</td>
</tr>
<tr>
<td>T37</td>
<td>Nopaline-strain</td>
</tr>
<tr>
<td>A136 NC</td>
<td>Octopine-strain</td>
</tr>
<tr>
<td>A281</td>
<td>Nopaline-strain</td>
</tr>
</tbody>
</table>
The strains ACH5 and T37 (Sciaky et al., 1978), C58 (Holsters et al., 1980), A136 NC (Chupeau et al., 1974) and A281 (Hood et al., 1986) have been described.

The following disarmed *A. tumefaciens* strains which are binary construct host strains and vectors were used to transform *B. napus* and *A. thaliana*, and *E. coli* strains used for triparental mating.

<table>
<thead>
<tr>
<th>Strain Description</th>
<th>Resistances (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB101::pRK2013</td>
<td><em>E. coli</em> (helper strain) Km 50</td>
</tr>
<tr>
<td>C58C1 (pGV3850)/pBI121</td>
<td><em>A. tumefaciens</em> Rif 50, Km 50</td>
</tr>
<tr>
<td>LBA4404 (pAL4404)/pBin19</td>
<td><em>A. tumefaciens</em> Rif 50, Km50</td>
</tr>
<tr>
<td>C58C1 (pGV2260)</td>
<td><em>A. tumefaciens</em> Rif 50, Amp10</td>
</tr>
<tr>
<td>DH5::pBI121.1</td>
<td><em>E. coli</em> (GUS) Km 50</td>
</tr>
<tr>
<td>HB101::pBin19</td>
<td><em>E. coli</em> Km 50</td>
</tr>
</tbody>
</table>

Km: kanamycin, Ap: ampicillin, Rif: rifampicin

pRK2013 (Ditta et al., 1980), pGV3850 (Zambrisky et al., 1983), pAL4404 (Hoekema et al., 1983), pGV2260 (Deblears et al., 1985) have been described. The binary plasmid pBin19 (Bevan, 1984) carries a NPT II (neomycin phosphotransferase II) gene which confers resistance to kanamycin, under control of the NOS promoter, whilst pBI121 (Jefferson et al., 1987) also contains a GUS (β-glucuronidase) coding sequence driven by the CaMV 35S promoter.

### 2.1.2 Growth of Bacterial Cultures

Bacterial liquid cultures were started from glycerol stocks which were kept at -80°C, or from bacterial colonies on plates stored at 4°C. In the case of glycerol stocks, a piece of frozen stock was removed using a flame-sterilized and cooled scalpel blade and placed into appropriate volume of NB (nutrient broth) or NZY (Appendix I) medium containing selective antibiotics. Cultures were grown overnight, or until the required amount of growth had occurred, in a shaking incubator at 37 °C for *E. coli*, at 28 °C for *A. tumefaciens*. Single colonies were picked using a flamed and cooled bacterial loop or a sterile pipette tip, then
inoculated into culture medium. Single colonies were obtained by streaking cell suspensions from an overnight culture onto agar-solidified medium (NA) using a flame-sterilized bacterial loop. Media was cooled to around 50 °C before adding antibiotics and pouring into 9 cm petri dishes. Streaked plates were incubated upside down at 37 °C or 28 °C overnight or until colonies appeared. All bacterial cultures were handled within a laminar air flow cabinet.

2.1.3 Storage of Bacterial Cultures
Cultures were maintained for short periods of time (1 month) as single colonies on sealed plates of solid culture media at 4°C in a fridge. For longer periods of storage, glycerol stocks of cultures were made from overnight cultures mixed 1:1 with a solution of 60% glycerol in nutrient broth in a 2 ml cryogenic tube. Stocks were flash-frozen by dropping into liquid nitrogen and stored at -80 °C indefinitely.

2.1.4 Antibiotics Used In Bacterial Cultures
Antibiotics were used for selection of plasmids, or bacterial strains carrying resistance genes. All antibiotics were supplied by Sigma Chemical Co. Rifampicin was dissolved in methanol, and the remainder in distilled water. All antibiotics were sterilized by filtration (0.22 μm pore size) and then stored at -20 °C, but relatively stable kanamycin was stored at 4 °C. The following antibiotics were commonly used at the given concentrations.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Stock (mg/ml)</th>
<th>Concentration For Selection (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E. coli</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>12.5</td>
<td>12.5</td>
</tr>
</tbody>
</table>
2.1.5 Conjugation of Plasmids from *Escherichia coli* to *Agrobacterium tumefaciens*

This method is as described in Draper et al. (1988). Plasmids used for plant transformation were based on binary vectors carrying a wide host range RK2 origin of replication allowing maintenance in *E. coli* and *A. tumefaciens*. Mobilization was achieved by a single triparental mating using helper functions supplied by pRK2013 carrying mobilization (mob) and transfer (tra) genes (Ditta et al., 1980). Overnight cultures of three strains, the donor *E. coli* strain, the recipient *Agrobacterium* host and the helper strain (HB101::pRK2013) were mixed (100 µl each) by spreading onto a NA (nutrient agar) plate with a flamed glass spreader. Plates were then incubated overnight at 28 °C. Two streaks from each plate were resuspended in 500 µl NB (nutrient broth) and streaked out on NA plates containing selective antibiotics both for *Agrobacterium* (i.e. selection against *E. coli*) and the presence of the introduced recombinant binary plasmid. Plates were incubated for 2 days at 28 °C, during which time single colonies of transconjugant *Agrobacterium* generally appeared. Southern hybridizations on total *Agrobacterium* DNA (see sections 2.1.6 and 2.3.6) were used to confirm the success of triparental matings.

2.1.6 Isolation of Total Nucleic Acids from *A. tumefaciens*

This method is as described in Draper et al. (1988). A single colony of *Agrobacterium* was picked into 5 ml of NB containing selective antibiotics and grown overnight at 28 °C. 1.5 ml of cultures were transferred to microfuge tubes and pelleted by centrifugation for 5 minutes. After removing the supernatant, the pellet was resuspended in 300 µl TE (Appendix III), to which was then added 100 µl of 5% Sarkosyl. After mixing, 150 µl of 5 mg/ml pronase was added and incubated at 37 °C for 1 hour. The pronase-treated cell suspension was then mixed with 500 µl of phenol : chloroform, by repeatedly passing through a 1 ml pipette tip in order to shear the viscous mass of membranes present in the bacterial lysate. After centrifugation for 5 minutes at

24
12,000 rpm, the aqueous layer was removed to a new tube and this treatment was repeated three times by adding 500 μl phenol : chloroform. The volume of the aqueous phase was then estimated and 0.05 volumes of 5 M NaCl and 3 volumes of cold (-20 °C) ethanol added. After leaving 1-2 hours at -20 °C, nucleic acid was collected by centrifugation for 10 minutes at 12,000 rpm and the supernatant discarded. The pellet was rinsed in 250 μl of 80% ethanol, dried by vacuum desiccation and the DNA pellet resuspended in 50 μl of sterile distilled water. 14 μl was digested with relevant restriction enzymes, loaded onto a 0.8% gel, and the gel subjected to Southern hybridization essentially as described in section 2.3.6.

2.2 Plant Tissue Culture and Transformation

2.2.1 General Tissue Culture Conditions
All tissue culture work was carried out in a laminar air flow cabinet (John Bass Ltd.) using IMS (industrial methylated spirits) and flame-sterilized dissection equipment to prevent contamination. Sterile 9 cm plastic Petri dishes (Sterilin Ltd.) were used to contain explants. Nescofilm (Sigma) or Cling Film were used to seal the plates. Murashige and Skoog (1962) medium (MS) (see Appendix II) was used in all experiments as a basal medium. Cultures were maintained in a culture room at a temperature of 26 °C, a photoperiod of 16 hours light/8 hours dark and a light intensity of 40 μEm⁻²s⁻¹ provided by cool white fluorescent light.

2.2.2 Growth of sterile seedlings
B. napus cv Topaz and B. napus cv Cobra were used in preliminary regeneration and transformation experiments. B. napus cv Westar was used in sense and antisense transformation experiments. Seeds of B. napus were surface sterilized in 20% (v/v) of Domestos (commercial bleach) for 20 minutes. The seeds were then rinsed in sterile tap water three times using sterile sieves and sown either in sterile Kilner Jars (Ravenhead) containing vermiculite (Vermalite, Grade V3) wetted with Hoagland’s solution (Appendix II) or in
Magenta vessels (Sigma) containing MS medium supplemented with 3% sucrose and 0.7% agar (Technical standard, Oxoid). Seeds were germinated at 26°C in a 16 hours light / 8 hours dark photoperiod and at a light intensity of 40 μEm⁻²s⁻¹.

The *A. thaliana* ecotypes "C24" and "Landsberg erecta" used in this study were obtained from Dr. Bernard Mulligan, University of Nottingham, UK. *Arabidopsis* seeds were vernalised for 3-4 days at 4 °C before *in vitro* aseptic germination. The seeds were surface sterilized in 10% (v/v) Domestos for 10 minutes then rinsed three times with sterile tap water using sterile sieves. Seeds were germinated in 9 cm petri dishes containing MSO (MS+3% sucrose+0.7% agar medium without plant growth regulators) and maintained in the same conditions as *B. napus*.

### 2.2.3 Growth of plants

Seeds of *B. napus* were sown in Vacapots (H. Smith Plastics Ltd.) containing a peat-based compost : vermiculite (3:1) mixture and seedlings were established in the growth cabinet with a day/night temperature of 25/15°C, 16 hours light/8 hours dark photoperiod. 15-day old seedlings were transferred to 12 cm pots containing same potting mixture.

*A. thaliana* seeds were sown in seed trays in a peat-based compost : perlite (Silvaperl, standard grade) (3:1) mixture. Seed trays were placed in a 16 hour photoperiod and a temperature of 22 °C.

### 2.2.4 Tissue culture media

Murashige and Skoog (1962) medium (MS) was used as a basal medium (Appendix II). MS (Flow Laboratories) was solidified with 0.7% agar (Technical standard, Oxoid) and contained 3% sucrose. The pH was adjusted to 5.7 with 1 M KOH and the medium was sterilized by autoclaving at 121°C for 20 minutes. Growth regulators were added to the medium prior to autoclaving.
2.2.5 Plant Growth Regulators

Plant growth regulators were obtained from Sigma Chemical Co. Auxins were dissolved in 50% ethanol. Cytokinins were dissolved in 1 ml of 1 M HCl and 99 ml of distilled water added. They were filter-sterilized and stored in Universal bottles at 4°C up to 1 month. The following growth regulators were used to improve plant regeneration in vitro.

<table>
<thead>
<tr>
<th>Plant Growth Regulators</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AUXINS</strong></td>
<td></td>
</tr>
<tr>
<td>Indole-3-acetic acid (IAA)</td>
<td>175.2</td>
</tr>
<tr>
<td>Indole-3-butyric acid (IBA)</td>
<td>203.2</td>
</tr>
<tr>
<td>Naphthaleneacetic acid (NAA)</td>
<td>186.2</td>
</tr>
<tr>
<td>2,4-dichlorophenoxyacetic acid (2,4-D)</td>
<td>221.0</td>
</tr>
<tr>
<td><strong>CYTOKININS</strong></td>
<td></td>
</tr>
<tr>
<td>6-benzylaminopurine (BAP)</td>
<td>225.2</td>
</tr>
<tr>
<td>Kinetin</td>
<td>215.2</td>
</tr>
<tr>
<td>N6-(2-isopentenyl) adenine (2iP)</td>
<td>203.3</td>
</tr>
</tbody>
</table>

2.2.6 Antibiotics for Plant Transformation

Kanamycin was used to select transformed plants in vitro. Cefotaxime, carbenicillin, augmentin and vancomycin were used to kill Agrobacterium after co-cultivation. All four antibiotics were dissolved in distilled water and filter-sterilized (with 0.22 μm pore size). Augmentin was made up freshly each time since it is very unstable. The concentration of antibiotics used are shown below:

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>N. tabacum</th>
<th>A. thaliana</th>
<th>B. napus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin</td>
<td>100</td>
<td>50</td>
<td>15</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>250</td>
<td>-</td>
<td>250</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>500</td>
<td>-</td>
<td>500</td>
</tr>
<tr>
<td>Augmentin</td>
<td>-</td>
<td>400</td>
<td>400</td>
</tr>
</tbody>
</table>
2.2.7 Transformation of *Nicotiana tabacum*

Tobacco plants were transformed using a leaf disk method described in Draper *et al.* (1988). Young (just fully expanded) tobacco leaves taken from greenhouse grown plants were sterilized in 10% bleach for 15 minutes. The leaves were then rinsed in sterile tap water four times. After removing bleach-damaged areas, the leaf was cut into 0.5-1 cm squares avoiding the major leaf veins. Explants were placed onto MSD4x2 (MS, 1 mg/l BAP, 0.1 mg/l NAA) solid medium at a density of 6-7 disks per 9 cm Petri dish. Explants were placed into a dish containing a 1:50 dilution (in MSD4x2) of overnight culture of *Agrobacterium* strain for 15 minutes. After removal of excess liquid, the leaf disks were replaced on the same MSD4x2 plates and co-cultivated for 2 days in culture room (see section 2.2.1). After co-cultivation, explants were transferred to regeneration/selection medium i.e. MSD4x2 containing 100 μg/ml kanamycin and 250 μg/ml cefotaxime. They were incubated in the same culture room until kanamycin resistant shoots regenerated from the callus around the explants. Kanamycin resistant shoots, which appeared green, were excised and transferred to Magenta vessels (Sigma) containing MSO medium with antibiotics (100 μg/l kanamycin and 250 μg/l cefotaxime) for rooting. Rooted plantlets were then transferred to soil, sealed in a plastic bag to provide a humid condition for the first 2-3 days and then grown as normal in the growth room.

2.2.8 Regeneration and Transformation of *A. thaliana*

Wild-type *A. thaliana* were grown in the greenhouse in seed trays in a peat based potting-mix on a 16 hour photoperiod and maintained at 25 °C. Immature cotyledon explants used to initiate cultures were obtained from siliques that were at the final stages of maturity but before desiccation and silique break. Siliques were surface sterilized for 20 minutes in 20% bleach followed by 3 rinses with sterile tap water. Immature seeds were dissected and placed on MSO medium to germinate. Cotyledons were dissected from the maturing seeds as described by Patton and Meinke (1988). For this purpose, *A. thaliana* seeds
were vernalized for 3-4 days at 4 °C before sterilization in 10% bleach for 10 minutes. After rinsing with sterile tap water they were sown on MS0 medium and incubated in the culture room 3-4 days after germination. Cotyledons were removed from germinated seedlings or from immature seeds without including any of the adjacent meristematic tissue. Cotyledon explants were floated in liquid MS0 medium within a sterile sieve + plate, until 40-50 cotyledons had been collected. They were then transferred to 1/10,1/50 and 1/100 dilutions (in MS0) of an overnight Agrobacterium culture for 3-180 minutes. The cotyledons were dried by placing the sieve on sterile filter papers and transferred to regeneration medium (MS medium supplemented with 0.1-0.4 mg/l NAA and 1.0 mg/l BAP) at a density of 25-30 explants per 9 cm plate. After 2-3 days co-cultivation, the explants were transferred to selection medium (regeneration medium supplemented with 50 µg/ml kanamycin) containing 500 µg/ml vancomycin or 400 µg/ml augmentin. If the plants were overgrown by Agrobacterium during co-cultivation, the explants were then washed in sterile water and blotted on a sterile filter paper. The cotyledons were subcultured every two weeks to selective media plates. Kanamycin resistant shoots were excised from the green callus and placed in MS0 medium with a reduced amount of sucrose (2%) for rooting. Plants were subsequently transferred to soil (compost and perlite) and covered with plastic bags for 5-6 days then grown in a growth cabinet (22°C temperature, 16 hour photoperiod) without covers.

2.2.9 Regeneration and Transformation of B. napus

B. napus cotyledonary explants were investigated using various explant types and plant growth regulators. 2/3 distal cotyledon, cotyledon + petiole and immature cotyledon explants were used. 2/3 distal cotyledon explants were excised from 5-day old seedlings that were grown in sterile conditions. Cotyledons were removed from the seedlings and placed onto a sterile tile or a Petri dish. The basal end of the lamina was removed and cut surfaces of the explants were embedded into solid MS medium supplemented with growth
regulators. The plating density was 5 explants per 9 cm plate and they were subcultured to fresh medium every two weeks. Cotyledon + petiole explants were also obtained from 5-day old sterile seedlings. Cotyledonary explants which included a 2-3 mm long petiole were excised from seedlings, and then dipped into solid MS medium at a density of 5 or 10 explants per plate. They were then inoculated with overnight culture of A. tumefaciens. A 5 ml overnight culture of A. tumefaciens was pelleted by centrifugation for 5 minutes at 12,000 rpm then resuspended in 10 ml of liquid MS0 medium (Moloney et al., 1989). Cut surfaces of petioles were immersed into this bacterial suspension for 5 seconds and they were immediately returned to the same medium. The cotyledons were co-cultivated with Agrobacterium for 2-3 days. After co-cultivation, the cotyledonary explants were transferred to regeneration medium supplemented with 15 μg/ml kanamycin and 400 μg/ml augmentin. They were subcultured to fresh medium every two weeks.

Immature cotyledon explants for culture initiation were dissected from seed pods that were at the final stages of maturity but before desiccation had commenced (ten weeks old B. napus cv Topaz, 18-20 days after pollination). Seeds pods containing immature seeds were surface sterilized in 10 % (v/v) bleach for 15 minutes then rinsed three times with sterile tap water. Immature seeds were separated from the pods and then immature cotyledons were dissected carefully using fine forceps and a scalpel. During this process all auxiliary buds were removed from the explants. Dissected immature cotyledons were plated on solid MS medium containing growth regulators at a density of 10 explants per plate. Regenerated shoots were transferred to MS0 medium for rooting and finally transferred to soil and kept under plastic covers for first 2-3 days.
2.3 Restriction Digestion and Southern Blot Analysis of Plant DNA

2.3.1 Isolation of Plant DNA

Plant DNA was isolated using a CTAB (Cetyl trimethylammonium bromide) extraction method, as described by Draper et al. (1988). Leaf tissue was flash frozen in liquid nitrogen and ground to a fine powder with alumina in a mortar and pestle. This powder was transferred to a 50 ml polyallomer centrifuge tube and mixed with 1 ml/g of 2 x CTAB buffer (Appendix III) at 60°C and incubated at 60 °C for 30-60 minutes. An equal volume of chloroform:octanol (24:1) was added, mixed gently and centrifuged at 12,000 rpm for 5 minutes. The aqueous phase was then removed to a new tube and 0.1 volume of 10% CTAB (Appendix III) at 60 °C was added. After re-extraction with chloroform:octanol, DNA was precipitated with an equal volume of cold (-20 °C) propan-2-ol and collected by centrifugation at 12,000 for 10 minutes. The pellet was washed in 80% ethanol and dried before resuspension in sterile distilled water.

2.3.2 Digestion of DNA with Restriction Endonucleases

Restriction enzymes were obtained with an accompanying buffer, and used according to manufacturer's recommendations. Plasmid or plant DNA was digested with an appropriate amount of restriction enzyme and in the appropriate buffer (and where needed 0.5 μl of 0.5 mg/ml RNase), and the total volume of reaction was made up with sterile distilled water. Up to 2 μg of plasmid DNA or 10 μg of plant genomic DNA was incubated for 2-16 hours at 37°C.

2.3.3 Fractionation of DNA by Agarose Gel Electrophoresis

Agarose concentrations varied between 0.8 and 1.2% depending on the expected size of the fragment. Agarose was completely dissolved in TAE buffer (Appendix III) by boiling with periodic swirling, and ethidium bromide (0.1 μg/ml) added. Gels were allowed to solidify for 30-40 minutes at room temperature. 3 μl of loading buffer (Appendix III) was added per 10 μl of the nucleic acid sample.
and each was loaded into the separate wells of the gel. Electrophoresis was performed in TAE buffer at 20-100 Volts, with appropriate molecular weight markers (e.g. lambda-DNA and 1kb Ladder, BRL) included on the gel. DNA was stained by ethidium bromide and visualized by fluorescence under U.V. light on a transilluminator.

2.3.4 Preparation of DNA Probe Stocks
Probes were prepared by either restriction digestion of plasmid DNA or by the polymerase chain reaction (PCR). In the first method, 1.5 µg of plasmid DNA was digested with an appropriate restriction enzymes to release the insert for radiolabelling. Fragments were separated by agarose gel electrophoresis on a low melting point gel (0.8%, Sigma). The correct size band was identified using a U.V. transilluminator, excised from the gel and placed into a microfuge tube. In second method, a DNA fragment was amplified from plasmid DNA by PCR (see section 2.7.4) and recovered as a gel slice from a low melting point gel.

2.3.5 Preparation of Herring-Sperm DNA
10 mg of herring-sperm was dissolved partially in 1 ml distilled water by heating at 65 °C. After that partially dissolved herring-sperm was passed through a range of needles (big to small) for 4-5 times using an appropriate syringe to shear the DNA. They were stored in the microcentrifuge tubes at -20 °C and boiled 5-10 minutes to denature the DNA before adding to hybridization solution.

2.3.6 Southern Blotting and Hybridization
After electrophoresis, the gel was transferred to a plastic box and shaken in 2-3 gel volumes of depurinating solution for 7 minutes, denaturing solution for 30-120 minutes and neutralizing solution for 30-120 minutes (all three solutions are described in Appendix III). The gel was rinsed in distilled water between treatments. The blotting apparatus was assembled as follows: a tray was filled
with 20xSSC (Appendix III) to 0.5 cm below the top of the sponge. A sheet of Whatman 3MM paper was placed on the sponge and allowed to soak up the 20xSSC. The whole tray was covered by a piece of cling film and then a gel-sized hole cut in the cling film. The gel was orientated by cutting off a corner and then the gel placed in the hole cut into the cling-film, avoiding trapping any air. The Hybond-N membrane (Amersham) was marked with pencil to allow orientation and carefully placed onto the gel. Any air bubbles between the membrane and gel were removed. Three layers of 3MM filter paper were cut to the size of the gel, wetted with 20xSSC, then laid on top of the membrane. A further three pieces of dry 3MM filter paper were placed onto the wet 3MM filter papers. A stack of paper towels was then placed over the gel and a 500 ml medical flat bottle filled with water was placed the top. The assembly was left overnight to allow capillary transfer of buffer from the tray to the towels through the gel. After blotting the membrane was removed and rinsed in 3xSSC, dried at 65 °C for 20-30 minutes and the DNA bound to the filter by 75 seconds U.V. cross linking (1.2 joules, UV Stratalinker 2400).

To block non-specific binding, the cross-linked filter was pre-hybridized in 20 ml of hybridization solution (Appendix III) containing 1 ml of 10 mg/ml boiled and sheared herring-sperm DNA (see section 2.3.5) at 65 °C for 1-2 hours. Meanwhile the DNA probe fragments were labelled with $^{32}$P using a random primed method (oligolabelling). The oligolabelling reaction consisted of 3 μl of oligolabelling buffer (OLB), 0.6 μl of 10 mg/ml BSA (bovine serum albumin), 0.6 μl of 1unit/μl Klenow fragment (Pharmacia), 1.0-1.5 μl of 10 μC/ml $[^{32}$P] dCTP (Amersham), 10 ng of DNA probe stock (probe stock was boiled for 5 minutes prior to addition to labelling reaction) and dH$_2$O to 15 μl. The labelling reaction was carried out at 37 °C for 1-2 hours and then the reaction was stopped with the addition of 65 μl of stop solution (Appendix III). For quantitation of total input radioactivity (cpm), 1 μl of the sample was spotted onto the centre of a Whatman GF/C glass-fibre disk (2.4 cm). Incorporation of radioactivity was measured by taking a 1 μl aliquot and mixing with 500 μl of herring-sperm DNA (500 μg/ml in
20 mM EDTA) and adding 125 μl of 50% TCA to precipitate the nucleic acids. The solution was filtered to collect the precipitate through a GF/C disk in a filter tower by washing 2x with 5 ml of 10% TCA and 1x with 5 ml of IMS (industrial methylated spirit). Radioactivity was measured by liquid scintillation counting. The unwashed filter gave the total input radioactivity and the washed filter gave the incorporated counts. Incorporation (%) and specific activity (cpm/μg) of the probe DNA was then calculated. Generally, 4-6 x 10^7 cpm (60-70%) incorporation was obtained. After pre-hybridization the labelled-probe was boiled for 5 minutes and added to the hybridization solution. Hybridization was carried out at 65 °C overnight and then the filter was washed at 65°C twice for 15 minutes each with wash A and with wash B (Appendix III). The filter was then air-dried between Whatman 3MM filter paper at room temperature before wrapping in "Saran Wrap" and exposed to X-ray film in a cassette at -80 °C.

2.4 Cloning of DNA Into Plasmid Vectors

2.4.1 Bolling Preparation of Plasmid DNA

This method is taken from Sambrook et al. (1989). Bacterial cultures were grown overnight under antibiotic selection. 1.5 ml of culture was then pipetted into microcentrifuge tubes and centrifuged for 1-2 minutes at 12,000 rpm. After removing the supernatants, 50μl of 25% sucrose, 300 μl of M-STET were added to the pellet which was then resuspended by vortexing. 25 μl of 20 mg/ml lysozyme were added and vortexed to allow the lysozyme to function. Within 5-10 seconds the tubes were boiled for 45 seconds and then centrifuged for 10 minutes at 12,000 rpm. After removing the gelatinous masses with a pipette tip, 1/4 volume of 10 M ammonium acetate and 2 volumes of cold (-20 °C) propan-2-ol were added. Microfuge tubes were inverted to mix and left at room temperature for 10 minutes to precipitate the nucleic acids. They were then centrifuged for 10 minutes and the supernatant discarded. 250 μl of 80% ethanol was added and the tubes inverted and centrifuged 1 minute. Washing the pellets in 80% ethanol was repeated. After
removing the supernatants, the tubes were dried in a vacuum desiccator and the DNA pellets resuspended in 30 μl distilled water.

2.4.2 Large Scale Preparation of Plasmid DNA

An alkaline lysis method was used according to Sambrook et al. (1989). 50 ml of bacterial culture was grown overnight and centrifuged for 10 minutes at 3600 rpm. The pellet was then resuspended in 1/10 volume of lysis buffer (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0). The suspension was mixed with 2 volumes of alkaline SDS and the tube left on ice for 5 minutes. 1.5 volumes of 3 M potassium acetate pH 5.2 was then added to the suspension and left on ice for 5 minutes to precipitate protein and chromosomal DNA. After centrifugation at 3600 rpm for 10 minutes, the supernatant was filtered through polyalomer wool into a new tube, and the plasmid DNA precipitated by the addition of 1 volume of cold propan-2-ol. After leaving the tube at room temperature for 10 minutes the nucleic acids were collected by centrifugation for 10 minutes at 3600 rpm. The supernatant was removed and the tube left inverted to remove the remainder of the supernatant. The pellet was resuspended in 200 μl of sterile distilled water and incubated with 5 μl of 10 mg/ml RNase at 37 °C for 30 minutes. Three phenol : chloroform extractions were performed each by the addition of 500 μl of phenol : chloroform (1:1), vortexing for 15 seconds and centrifugation for 5 minutes and removal of the aqueous layer to a new tube. After the last phenol : chloroform extraction 1/20 volume of 4 M sodium acetate pH 6.0 and 2 volumes of cold ethanol were mixed with the aqueous phase and then left at -20 °C for 30 minutes. The suspension was then centrifuged for 10 minutes and the pellet resuspended in 100 μl sterile distilled water (or TE) after vacuum-drying.
2.4.3 Purification of DNA From Agarose Gels

Electroelution and "Geneclean" methods were used to elute DNA from agarose gels. After electrophoresis of restriction digested DNA (see 2.3.2 and 2.3.3), the DNA band was cut out using a scalpel and placed into the electroelution apparatus. Electroelution was carried out within stop buffer (3 M sodium acetate, bromophenol blue, pH 7.9) at 140 volts for 15 minutes and salt was collected into microfuge tubes using a syringe. This process was repeated three times. An equal volume of butan-2-ol was then added, mixed, centrifuged for 5 minutes and the top layer was removed. After repeating this process three times, until the total volume of the aqueous layer was reduced to 400 μl, an equal volume of phenol/chloroform was added, vortexed, then left at room temperature for 10 minutes. Following a further centrifugation for 5 minutes, the top layer was transferred to another tube. DNA was precipitated using the method described in section 2.4.1.

The "Geneclean" method was performed using the "Geneclean II" DNA purification kit (Bio 101 Ltd.) according to manufacturer’s instructions.

2.4.4 Dephosphorylation of Restriction Fragments

Calf intestinal alkaline phosphatase (CIP) (BCL) removes 5'-phosphate groups from linear DNA. Plasmid DNA (10 μg) was digested with the desired restriction enzyme. When digestion was complete, the sample was extracted with phenol : chloroform and precipitated with 2 volumes of ethanol. After recovering DNA by centrifugation, the DNA pellet was redissolved in 10 μl of sterile distilled water, and incubated with 0.05 units of CIP in CIP buffer at 37 °C for 30 minutes. The enzyme was inactivated by incubation at 65° for 45 minutes in the presence of 30 mM trinitriltriacetic acid. DNA was recovered by phenol : chloroform extraction and ethanol precipitation.
2.4.5 Ligation of DNA Fragments

Ligation was carried out using 40-60 ng of vector DNA and insert DNA, 3 μl of 5x ligation buffer (BRL) (Appendix III), 1 μl (2 units) of T4 DNA ligase (BRL) in a total volume of 15 μl. The ligation mix was left at room temperature for 1-2 hours or at 14 °C overnight before transformation of competent *E. coli* cells.

2.4.6 Transformation of *E. coli* with Plasmid DNA

Competent cells that were kept at -80 °C were thawed on ice. Then 100 μl of competent cells (strain 71-18), 10 μl of 10 x TCM buffer and 6 μl of the completed ligation reaction were mixed in a microfuge tube. This mixture was incubated on ice for 30 minutes. It was then heat shocked by incubation at 37 °C for 2 minutes. After incubation at room temperature for 10 minutes, 1 ml of Luria Broth (LB) was added and the tube incubated at 42 °C for 2 minutes. The cells were then incubated at 37 °C for 30 minutes, 4 ml of LB added, and shaken at 37 °C for 2-3 hours. After centrifugation, the pellet was resuspended in 100 μl of LB. Transformant colonies were produced by spreading this suspension on selective LA (Appendix I) plates and incubating at 37 °C overnight.

2.4.7 Identification of Recombinant Plasmids In Transformed Colonies

Some vectors (pTZ 18/19 and pBluescript) contain a multicloning site for the insertion of DNA within the β-galactosidase (*lacZ*) gene. Insertion of DNA into the multicloning site therefore disrupts the production of a functional β-galactosidase enzyme. When induced by IPTG (isopropyl-β-thiogalactopyranoside) this enzyme cleaves the indigogenic substrate X-GAL (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) forming a blue product easily visible in colonies. When *lacZ* is interrupted by an insert, recombinant colonies are white and can be distinguished from non-recombinants. For blue/white selection, 20 μl of 40 mg/ml IPTG in water, and 20 μl of 40 mg/ml X-GAL in dimethylformamide were spread onto plates prior to spreading cells.
2.4.8 Colony Hybridization

If colour selection could not be used to identify recombinant colonies, colony hybridization was performed. Whatman 3MM filter papers were placed into 15 cm Petri dishes and which contained respectively: (1) 0.5 M NaOH, (2) Tris-HCl, pH 7.4, (3) Tris-HCl, pH 7.4 and (4) 0.5 M Tris, 1.5 M NaCl. The Petri dishes were contained enough solution to just wet each filter paper. Nylon membrane (Hybond-N) was cut to the size of the culture plate and laid onto the plate for 2 minutes. The membrane carrying the colonies was then lifted and placed colony side up onto wetted-filters. The membrane was left 5 minutes in (1), 3 minutes in (2), 3 minutes in (3) and 5 minutes in (4) filters. After drying at 60 °C for 20 minutes, the filter was cross-linked on the U.V. transilluminator for 90 seconds. Hybridization was carried out as described in section 2.3.6. The putative recombinant colonies were grown in LB overnight. Plasmid DNA isolation and restriction digestion analyses carried out to confirm that the plasmid contained the correct insert.

2.5 Screening of Libraries

2.5.1 *B. napus* Anther cDNA Library

A *B. napus* cDNA library constructed from anthers of 1.2-1.8 mm buds by Scott *et al.* (1991) was screened. The library was constructed in lambda ZAP II (Stratagene) a bacteriophage lambda insertion vector which contains six unique cloning sites within the pBluescript SK* phagemid sequence and will accommodate cDNA inserts from 0-10 kb in size. The phagemid has the bacteriophage f1 origin of replication, allowing rescue of single stranded plasmid DNA which can be used for DNA sequencing by co-infection with the filamentous helper phage M13K07. The Bluescript + insert can be recovered from lambda Zap as a plasmid (i.e. do not need to subclone the insert into a plasmid).
2.5.2 Plating of Lambda Phage

XL1-Blue was used as a host *E.coli* strain since it contains the F' episome required for both colour selection and *in vivo* excision. The F' episome contains the genes for expression of the bacterial F' pili which are required for filamentous phage infection. The Tn10 tetracycline gene is also located on the F' episome, therefore, in the presence of tetracycline, the episome is selectively maintained. Firstly, the number of plaque forming units (p.f.u.) of the library was tested by trial platings using the method described below. The host strain XL1-Blue was grown overnight in NZY medium containing 0.2% maltose, 10 mM MgSO4 and 10 μg/ml tetracycline. 600 μl of these XL1-Blue competent cells were then mixed with the desired number of p.f.u (~5000 per 14 cm plate) from the library and incubated at 37 °C for 15 minutes. 6-7 ml of top agar, cooled to 48 °C, was mixed with the pre-incubated cells and the resulting mixture poured immediately onto solid pre-warmed NZY medium in 14 cm Petri dishes. The plates were incubated at 37 °C for 6-8 hours until the plaques of the desired size appeared.

2.5.3 cDNA Library Screening

After incubation at 37 °C, the plates were cooled to 4 °C to ensure that the top agar did not separate from the bottom agar during the lifting procedure. Hybond-N discs were laid onto the plates for 30 seconds, during which time the orientation of the disc was marked by pushing a needle through the disc and marking the position of the holes on the base of the Petri dish using a marker pen. Membranes were placed plaque-side-up on 3MM Whatman filter paper soaked in denaturing solution for 7 minutes then transferred to a second filter paper soaked in neutralizing solution for 7 minutes. The membrane discs were then rinsed in 2x SSC for 2 minutes and dried before cross-linking on an U.V. transilluminator. The filters were then probed with labelled-DNA essentially as described in section 2.3.6. Pre-hybridization was carried out at 60 °C in hybridization solution for 1-2 hours and then the [32p]-labelled probe was added.
and hybridized at 60 °C overnight. To remove unbound probe, filters were washed in wash solution A for 20 minutes at 60 °C and dried on filter paper before exposing to X-ray film. Positive plaques were spotted on the original plates by orientation and each cored into 1 ml of SM buffer (Appendix III) using a glass pipette. After leaving at 4 °C for 1 hour, the cores were used for rescreening. Using 9 cm petri dishes, rescreening was performed as described above. However, 200 µl of XL1-Blue competent cells were mixed with the desired number of p.f.u. (200 p.f.u. per 9 cm plate).

2.5.4 Screening of Arabidopsis Genomic Library

The Arabidopsis genomic library was constructed in Lambda Dash II (Stratagene) in Leicester by Michael Roberts. Lambda Dash II is a versatile genomic cloning vector that accepts inserts from 9-23 kb. LE392 was used as a host E. coli strain. After calculating the titer of the genomic library, lifting and screening were performed as explained in 2.5.3. Initially, about 100,000 p.f.u. were screened using four plates (20,000 p.f.u. per 14 cm plate).

2.6 DNA Sequencing

2.6.1 Preparation of Template

Transformed cells carrying the DNA to be sequenced were grown in 2xYT medium to a cell density of OD₆₀₀ = 0.5-0.8. 2 ml of culture was then infected with filamentous helper phage M13K07 at a multiplicity of infection of 10 by shaking at 37 °C for 1 hour. 400 µl of infected cells were then transferred to 10 ml of 2xYT medium supplemented with 20 µg/ml kanamycin (the helper phage carries kanamycin resistance) and grown at 37 °C overnight. The resulting culture produces phagemid DNA packaged as single stranded filamentous phage that is extruded from cells into the medium.

1.5 ml of the overnight culture was pelleted by centrifugation for 10 minutes, and the upper 1 ml of the resulting supernatant (bottom of the tube may contain cells) was transferred to a new tube to which was added 200 µl of 20%
PEG 8000 and 2.5 M NaCl to precipitate phage particles. The mixture was then inverted and left at room temperature for 20 minutes. The phage was collected by centrifugation for 10 minutes, then after removing the supernatant, it was centrifuged again for 5 minutes to remove all traces of PEG. The pellet was then resuspended in 100 µl of TE (10 mM Tris, 1 mM EDTA) and 50 µl phenol by leaving on ice for 10 minutes then vortexing. Single stranded DNA was stripped out of its viral protein coat by treatment with phenol since phenol removes proteins from nucleic acids. After leaving the suspension at room temperature for 10 minutes, it was vortexed and centrifuged for 5 minutes. 80 µl of top layer (aqueous phase) was removed to a new tube and an equal volume of chloroform / octan-1-ol added to remove any phenol. It was then centrifuged again and aqueous phase was transferred to a new tube. Nucleic acids were precipitated at room temperature for 10 minutes by adding 1/4 volume of ammonium acetate and 2 volumes of propan-2-ol. After centrifugation for 10 minutes at 12,000 rpm the pellet was washed twice with 80% ethanol. After drying the pellet in a vacuum desiccator, the DNA was resuspended in 40 µl of sterile distilled water.

2.6.2 Dideoxy Chain Termination DNA Sequencing
Sequencing was carried out using a T7 DNA polymerase sequencing kit (obtained from USB) which employs dideoxynucleotide chain-termination mixes, as described by Sanger et al. (1977). Annealing was carried out with 7 µl of template, 1 µl of T3 primer and 5 x reaction buffer by heating at 65 °C for 2 minutes then left in a beaker of water to cool to < 35 °C. Four termination (ddG, ddA, ddT, ddC) mixtures, 2.5 µl of each were pre-warmed at 37 °C. The labelling reaction was set up with 10 µl of annealed DNA mix, 1 µl of 0.1 M DTT, 2 µl of diluted labelling mix (7.5 µM dGTP, dCTP, dTTP), 0.5 µl of 10 µCi/µl [35S] dATP and 2 µl of diluted sequenase (1 µl of sequenase in 7 µl of enzyme dilution buffer) then incubated at room temperature for 5 minutes. 3.5 µl of the labelling reaction was transferred to each termination tube, mixed and incubated at 37 °C.
for 5 minutes. The reactions were then stopped by adding 4 μl of stop solution.
The samples were heated to 75 °C for 3 minutes just before loading onto the
polyacrylamide gel. Acrylamide gels were prepared by polymerizing 50 ml of
6% acrylamide (5.7% acrylamide, 0.3% bis-acrylamide in 7 M urea and 1 x TBE)
by the addition of 50 μl tetramethylethlenediamine (TEMED) and 50 μl of 250
mg/ml ammonium persulphate. This mixture was poured into Bio-Rad “Sequi-
Gen” sequencing plates and polymerized for 1 hour. The gel was pre-run to a
temperature of 50 °C at 2700 volt using 1 x TBE as a buffer. 2.5 μl of pre-heated
samples were loaded onto the gel. Electrophoresis was at ~2200 volts to
maintain temperature of 50 °C for 1.5-6 hours. The gels were dried onto
Whatman 3MM filter paper and exposed to X-RAY film at room temperature
overnight.

2.6.3 Analysis of Data
Sequence data was analysed on the Leicester University Computer Centre VAX
mainframe, using the Wisconsin Genetics Computer Group (GCG) program suite
(Devereux et al., 1984). Sequences were aligned using the program CLUSTAL
(Higgins and Sharp, 1989).

2.7 Analysis of Transformed Plants
2.7.1 Histochemical GUS (β-Glucuronidase) Activity Assay
GUS assays were performed according to Draper et al. (1988).
Fresh tissues and sections were placed in fixative solution (0.3% formaldehyde,
10 mM MES pH 5.6, 0.3 M mannitol) and vacuum infiltrated for about 1 minute.
They were then incubated for 30 minutes at room temperature, followed by
several washes in 50 mM NaPO₄ pH 7.0. The sections were transferred to 1-2
ml of X-Gluc (5-bromo-4-chloro-3-indolyl glucuronidase, Research Organics)
and incubated at 37 °C for 20 minutes to overnight, depending on the intensity
of the staining. 10 mg of X-Gluc was dissolved in 100 μl of dimethylformamide
and made up to 10 ml with 50 mM NaPO₄ pH 7.0. After staining, sections were
rinsed in 70% ethanol.

Histochemical GUS assays in anthers was performed according to Koltunow et al. (1990). Firstly, anthers were sliced into 2 mm pieces and fixed in 0.1 M NaPO$_4$ buffer (pH 7.0) containing 0.1% formaldehyde, 0.1% Triton X-100 and 0.1% β-mercaptoethanol by vacuum infiltration for 15 minutes. They were then rinsed extensively with 0.1 M NaPO$_4$ (pH 7.0) containing 0.1% β-mercaptoethanol, and with 0.05 M NaPO$_4$ + 0.1% β-mercaptoethanol. The anther slices were incubated at 37 °C in 0.05 M NaPO$_4$ (pH 7.0) containing 0.1% Triton X-100, 0.1% β-mercaptoethanol and 0.001 M X-Gluc. After incubation, anthers were fixed in 5% formaldehyde, 5% acetic acid and 20% ethanol for 2 hours. Chlorophyll was cleared by shaking in 50% ethanol for 2 hours and in 100% ethanol overnight.

2.7.2 Fluorimetric Assay for Quantitation of GUS Activity

Fluorimetric GUS assay was performed using 4-methylumbelliferyl glucuronide (4-MUG) as described by Draper et al. (1988). Protein was isolated by grinding plant material in GUS extraction buffer (see Appendix III). 10 μl protein extract was then added to 500 μl of 37 °C pre-warmed assay buffer (extraction buffer supplemented with 1 mM MUG) and continuing the incubation at 37 °C. 20 μl aliquots from the reaction were added into wells of an opaque microtitre plate containing 180 μl of 0.2 M Na$_2$CO$_3$ stop solution after 0, 5, 10 and 20 minutes. The fluorescence of the GUS-catalysed breakdown product of MUG was measured using a fluorimeter. Fluorescence values were standardized relative to the quantity of total protein present in the reaction.

2.7.3 Bradford Assay for Quantitation of Protein

Protein concentrations were determined by the method of Bradford (1976). 100 μl distilled water, 100 μl Bradford's solution (see Appendix III) and 1-10 μl of the protein sample were combined in the wells of the microtitre plate. Solution of bovine serum albumin in the range 0 to 1.0 mg/ml were used as standards to
calculate protein concentration. Protein was quantitated using a Dynatech MR5000 microtitre plate reader.

2.7.4 PCR Analysis of Transformed Plants

A leaf disc was removed from the plant using the lid of a sterile 1.5 ml microcentrifuge tube and DNA extracted by the method of Edwards et al. (1991). The leaf discs were frozen on dry-ice and mashed with a disposable mortar for 15 seconds. 400 µl of extraction buffer (Appendix III) was then added and vortexed for 5 seconds. After centrifugation for 1 minute at 12,000 rpm, 300 µl of the supernatant was mixed with 300 µl of propan-2-ol and incubated at room temperature for 2 minutes. This mixture was then centrifuged at 12,000 rpm for 5 minutes and supernatant discarded. After vacuum drying, the pellet was resuspended in 200 µl TE. 1/100 th of the resulting DNA solution was used in a polymerase chain reaction (PCR), using primers at a concentration of 1 µM. Polymerase chain reactions were set up as follows: 2 µl of plant DNA (or 4 µl of plasmid DNA), 2 µl of 78 µg/ml primers, 1.8 µl of 10 x PCR buffer (Appendix III), 0.3 µl of 5 u/µl Taq DNA polymerase I (Cambio) and sterile distilled water to volume of 20 µl. Thirty amplification cycles were performed, each consisting of a 1 minute 20 second denaturation step at 94 °C, a 2 minute annealing step at 57 °C and a 3 minute extension step at 72 °C. The PCR products were analysed by electrophoresis on an agarose gels.

The following primers were used in PCR reactions:-

M13 20' (5' CGTTGTAAGACGGCACGGCCAG 3'),
RSPL (5' CACACAGGAACAGCTATGACC 3') which hybridize to pBin19,
5'-126 (5' GGATCCAACCAGGGCGACGCACACTG 3')
and 3'-240 (5' GGAAGCTATGTAACGCCCTTCAGCACAG 3') which hybridize to the B.napus A9 cDNA. The position at which these primers anneal to the constructs is indicated in Figure 6.1 (see Chapter 6).
2.7.5 Preparation of Primers for PCR

Oligonucleotide primers were mixed with 1/10 volume of 2M sodium acetate pH 7.0 and 3 volumes of ethanol. After leaving for 20 minutes at -80 °C, the mixture was centrifuged for 3 minutes at 12,000 rpm. The pellet was then washed with 80% ethanol before drying in a vacuum desiccator. The pellet was resuspended in sterile distilled water to 1/2 the original volume and an aliquot was diluted to 78 μg/ml for use in PCR reactions. To determine the density of the stock primer, 1 μl sample was diluted in 200 μl sterile distilled water and scanned to measure A260 using a scanning spectrophotometer (U.V. absorbance). The concentration of the primer was calculated as follows: $A_{260} \times 24 \times 200 = \mu g/ml$ (see section 2.7.7).

2.7.6 Extraction of Plant RNA

Total RNA was extracted using a method described by Verwoerd et al. (1989). *B. napus* RNA was isolated from buds of length <1 mm to >5 mm and tobacco RNA from anthers dissected from 10-12 mm long buds. The bud and anther samples were put into a microcentrifuge tube and ground immediately to a fine powder in liquid nitrogen using a micro-homogenizer. 500 μl of hot (80 °C) phenol : RNA extraction buffer was added and the mixture vortexed 30 seconds. After adding 250 μl of chloroform : octanol (24:1), it was again vortexed for 20 seconds and centrifuged for 5 minutes. The aqueous top layer was then transferred to a new cooled tube and mixed with 1 volume of 4 M LiCl. After precipitating for 3-4 hours, the tubes were centrifuged at 4 °C for 10 minutes at 12,000 rpm. The pellet was dissolved in 250 μl of sterile DEPC-treated water by vortexing. 20 μl of 4 M sodium acetate (pH 6.0) and 2.5 volumes of ethanol were then added and the mixture left at -80 °C for 15 minutes. After centrifugation for 10 minutes at 12,000 rpm the RNA pellet was dried at room temperature and the pellet was resuspended in 20 μl of sterile DEPC-treated water.
2.7.7 Spectrophotometric Determination of Nucleic Acids

For quantitating the amount of DNA or RNA, readings were taken at a wavelength (U.V. absorbance) of 260 nm. This allows calculation of the concentration of nucleic acid in the sample. An absorbance \( A_{260} \) of 1 corresponds to approximately 50 µg/ml for double-stranded DNA, 40 µg/ml for single stranded DNA and RNA, and 24 µg/ml for an oligonucleotide.

2.7.8 Northern Gel Blotting and Hybridization

RNA gel blots were performed according to Fourney et al. (1988) using 10 µg RNA per lane. Firstly, all solutions and equipment used for RNA work were treated with 0.1% diethylpyrocarbonate (DEPC) to create RNase-free conditions. 1.2 g of agarose (Sigma) was dissolved in 10 ml of 10x MOPS [3-(N-morpholine) propanesulfonic acid] and 87 ml of DEPC-treated autoclaved water and cooled to 50 °C. 5.1 ml of 37% (v/v) formaldehyde was then added into the agarose solution, gently mixed, and then poured into a gel tray. The volume of the RNA sample was adjusted to 2.5 µl with DEPC-treated sterile water and mixed with 12.5 µl of sample buffer. After heating at 65 °C for 15 minutes, 1 µl of ethidium bromide was introduced into sample and loaded on the gel. The gel was electrophoresed in 1x MOPS/EDTA buffer at 60 V for 4-5 hours. The gel was then soaked for 10 minutes in 0.05 M NaOH made up in 1x SSC and in 10x SSC for two 20 minutes periods by gentle shaking. The RNA was transferred to Hybond-N membrane in 10x SSC by capillary action using the same method as described for Southern blotting (section 2.3.6). RNA was cross-linked to the membrane by UV-irradiation for 75 seconds (UV Stratalinker 2400). Hybridization was carried out in 50% formamide, 6x SSPE (saline-sodium phosphate-EDTA), 5x Denhardts, 0.4% SDS and 6% polyethylene glycol (PEG 6000) at 42 °C essentially as described in section 2.3.6. The filter was washed at 42 °C only with wash A for 15-30 minutes.
2.7.9 Northern Dot Blotting
Dot blotting was carried out according to manufacturers' instructions. 5 µg of total RNA was mixed with 3 volumes of following solution: 500 µl of formamide, 162 µl of formaldehyde solution, 100 µl of 10x MOPS and incubated at 65 °C for 5 minutes to denature the RNA. The RNA sample was then chilled on ice and mixed with 1 volume of cold 20x SSC. The sample was then spotted onto Hybond-N (pre-wetted in 10x SSC) and the RNA was fixed to the membrane by 50 seconds U.V. cross-linking.

2.7.10 Staining for Pollen Viability and Scanning Electron Microscopy
The stain developed by Alexander (1969) was used to estimate the viability of pollen. This stain contains 10 ml of 95% ethanol, 10 mg malachite green, 25 ml glycerol, 5 g phenol, 5 g chloral hydrate, 5 mg Orange G, 50 mg acid fuchin, 2 ml glacial acetic acid and 50 ml of distilled water. Pollen was placed on a microscope slide, 20 µl of stain added, covered by a coverslip, then warmed over a small flame prior to microscopy. Viable pollen grains stained red and aborted pollen grains stained green. At least 100 pollen grains per plant were scored for viability.

For scanning electron microscopy, pollen grains from transformed and wild-type B. napus plants were placed on the aluminium pin stubs (Agar Scientific Ltd.) which was treated with sticky taps. After coated with gold, they were scanned with electron microscope (ISi - DS 130) and photograph taken.

2.7.11 Inheritance of Kanamycin Resistance in Transformed Plants
inheritance of kanamycin resistance from transformed B. napus and A. thaliana plants was investigated by germinating them on MS0 medium supplemented with appropriate amount of kanamycin. Seeds from selfed transformed plants and wild-type plants were grown on 50 mg/l kanamycin. After 7-10 days seedlings were scored for resistance to kanamycin. Kanamycin sensitive
seedlings bleached, whilst resistant seedlings remained green.

*B. napus* plants containing antisense or sense constructs were selfed or cross-pollinated with wild-type *B. napus* cv Topaz. For cross-pollination, wild-type anthers immediately post-anthesis were simply touched onto the stigma of the transgenic plant. Anthers were removed from the recipient flowers with a pair of forceps prior to pollination and cross-pollinated flowers were labelled to avoid confusion during seed collection. The progeny of the transformants, resulting from selfing and crossing were analysed by PCR to determine the segregation of the antisense gene.
CHAPTER 3

REGENERATION AND AGROBACTERIUM-MEDIATED TRANSFORMATION OF ARABIDOPSIS THALIANA

3.1 Introduction

*Arabidopsis thaliana* has found utility in studies of basic physiology and biochemistry as well as in plant molecular genetic manipulations and developmental biology research due to its small genome size, low chromosome number, short generation time (4-6 weeks), availability of many mutants and genetic maps, sexual self-compatibility and prolific seed production. However, more extensive use of *Arabidopsis* has been hampered because of difficulties in efficient and rapid regeneration and transformation procedures. Several methods for plant regeneration have been reported (Negrutiu *et al.*, 1978; Negrutiu and Jacobs, 1978; Gotto, 1979; Huang and Yeoman, 1984; Acedo, 1986; Feldmann and Marks, 1986; Gleddie, 1989). Transformed plants have been recovered from various explants such as leaf (Lloyd *et al.*, 1986), stem (An
et al., 1986), callus tissue (Zhang and Somerville, 1987), germinating seeds (Feldmann and Marks, 1987), root (Valvekens et al., 1988) and by using direct gene transfer to protoplasts (Damm et al., 1989). Despite these reports, the frequency of regeneration of transgenic plants is still low and requires at least a few months. Moreover, the long period of in vitro incubation during shoot regeneration may increase the possibility of somaclonal variation and changes in ploidy levels (Larkin and Scowcroft, 1981). Many reports have highlighted the high regeneration potential of cotyledon explants at various stages of development in maturing embryos or after seed germination (Duncan et al., 1985; Ranch et al., 1985; Patton and Meinke, 1988). The rapid and prolific shoot regeneration from immature cotyledons of Arabidopsis ecotype “Columbia” reported by Patton and Meinke (1988) indicated its regeneration potential.

Experiments aimed at improving the transformation of A. thaliana were initiated since large numbers of transgenic plants were required in order to evaluate antisense RNA constructs (see Chapter 6). As explained in Chapter 1, nucleotide conservation between B. napus and A. thaliana makes cross-hybridization possible between gene homologous from the two species. This was an important consideration because the intention was to target Arabidopsis genes for downregulation using antisense transcripts expressed from Brassica cDNA sequences. Initially, transformation of Arabidopsis with these antisense constructs was thought to be much more effective than transformation of B. napus since B. napus was reportedly difficult to work with. This report presents further studies on the development of a procedure for the rapid and prolific regeneration of shoots from cotyledon explants at various stages of maturity in Arabidopsis in two ecotypes i.e. “Landsberg erecta” and “C24”. Furthermore, the regeneration procedure developed in this study was used to establish a method for rapid production (within 2-3 weeks) of transgenic Arabidopsis shoots using disarmed A. tumefaciens.
3.2 Results

3.2.1 Effect of Explant on Regeneration

Immature cotyledon explants used to initiate cultures were obtained from seed pods that were at the final stages of maturity but before desiccation and seed pod break (about 12-13 days after pollination). Seed pods were sterilized as explained in materials and methods (Chapter 2). Immature cotyledon explants taken from seed pods of both ecotypes ("Landsberg erecta" and "C24") at various stages of maturity were studied for regeneration and transformation potential. The responses of both ecotypes were similar unless stated otherwise.

Seeds at three different stages of maturity (see Table 3.1) were examined for regeneration response on media containing various auxin (NAA and IBA) and cytokinin (BAP) combinations (Table 3.1, Figure 3.1). Cotyledons dissected from the most mature pods (stage I) produced the highest frequency of shoot regeneration. NAA gave the highest frequency of callus formation and adventitious shoot regeneration (Table 3.1, Figure 3.1). The cotyledons expanded rapidly in culture and produced callus at the cut surface within 3-4 days. Shoot regeneration was normally preceded by anthocyanin accumulation in the callus of "Landsberg" and shoot initials were observed in about 10 days. It has been suggested that rapid shoot regeneration from these explants may result in the induction of less somaclonal variation than in shoots produced from leaf or root explants which take longer to regenerate (Larkin and Scowcroft, 1981; Marton and Browse, 1991). A high proportion of explants produced callus (up to 100%) and shoot regeneration as high as 95% was obtained (Tables 3.1 and 3.2). Shoot regeneration was high on a wide range of media. A medium containing 0.1-0.4 mg/l NAA and 1-2 mg/l BAP was more suitable for initial callus growth followed by shoot regeneration (at least 8 shoots per explant) (Tables 3.1 and 3.2). Physiological abnormalities such as vitrification and reduced shoot elongation was common at 4 mg/l BAP. These abnormalities have been reported to be associated with a high concentration of BAP in shoot initiation medium (Tetu et al., 1987; Barghchi and Aiderson, 1989). Less mature
Table 3.1 Shoot regeneration from immature cotyledons of *Arabidopsis* ecotype "C24". Media: MS medium containing 1.0 mg/l BAP plus the following auxins (mg/l): 1) 0.1 NAA, 2) 0.4 NAA, 3) 0.1 IBA, 4) 0.4 IBA

<table>
<thead>
<tr>
<th>Media</th>
<th>Stage of maturity*</th>
<th>Stage of maturity</th>
<th>Stage of maturity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>Callus growth (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>100</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>100</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>35</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td>35</td>
<td>38</td>
<td>87</td>
</tr>
<tr>
<td>Shoot growth (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>95</td>
<td>77</td>
<td>64</td>
</tr>
<tr>
<td>2</td>
<td>92</td>
<td>62</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>32</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>0</td>
<td>33</td>
</tr>
</tbody>
</table>

* Stages of seed maturity: I= the first 3 seed pods maturing (browning) at the base of the inflorescence (most mature), II= the 3 seed pods above I (moderate mature), III= the 3 seed pods above II (least mature). Total explants/treatment= 50
Figure 3.1
Shoot regeneration from immature cotyledons of *A. thaliana* ecotype C24. Stages of seed maturity: I= the first 3 seed pods maturing (browning) at the base of the inflorescence (most mature), II= the 3 seed pods above I (moderate mature), III= the 3 seed pods above II (least mature). All four auxins were used with 1.0 mg/l BAP.
Table 3.2 Effect of BAP concentration on shoot regeneration from immature cotyledons (stage i) of Arabidopsis ecotypes “Landsberg” and “C24”. Medium: MS medium containing 0.1 mg/l NAA and above concentrations of BAP

<table>
<thead>
<tr>
<th>Growth*</th>
<th>BAP (mg/l)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>&quot;Landsberg&quot;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Callus</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Shoot</td>
<td>88</td>
<td>80</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>&quot;C24&quot;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Callus</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Shoot</td>
<td>85</td>
<td>78</td>
<td>68</td>
<td></td>
</tr>
</tbody>
</table>

* Percentage of regenerated explants (%). Total explants/treatment = 100

Table 3.3 Effect of cotyledon age on shoot regeneration from Arabidopsis explants ecotype "C24". Medium: MS containing 0.4 mg/l NAA and 1.0 mg/l BAP

<table>
<thead>
<tr>
<th>Growth response</th>
<th>Days after seed germination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Callus induction (%)</td>
<td>92</td>
</tr>
<tr>
<td>Shoot regeneration (%)</td>
<td>80</td>
</tr>
</tbody>
</table>

Total explants cultured/treatment = 50
cotyledons were more difficult to work with due to dehydration and their smaller size. Cotyledons from in vitro germinated mature seeds were also tested for shoot regeneration capacity. In this case, seeds were vernalized for 3-4 days at 4°C before sterilization as explained in materials and methods. They were germinated on MS0 medium and cotyledons were excised from germinated seedlings without including any of the adjacent meristematic tissue. 25-30 cotyledon explants were cultured per 9 cm Petri dish.

The results presented in Table 3.3 show that the capacity of the explants for shoot regeneration declined progressively with age. The frequency of shoot regeneration from cotyledon explants declined from 80% at 4-8 days post germination (dpg) to 8% after 16 dpg. Therefore, explants taken at 4-8 days after germination were optimum for shoot regeneration from cotyledons. It was also determined that shoot regeneration from cotyledon explants (4-8 dpg) was reduced from 82% to 16% by reducing incubation temperature from 26°C to 20°C. Therefore it was concluded that the optimum conditions for shoot regeneration involves the use of 4-8 dpg cotyledon explants cultured in MS medium supplemented with 0.1-0.4 mg/l NAA and 1-2 mg/l BAP incubated at about 26°C.

For rooting, in vitro grown shoots were from the explant and transferred to MS medium containing 0.8% agar, 1.5% sucrose and 1 mg/l NAA for 4 days. Subsequently, shoots were transferred to root elongation medium (1/2 MS, 0.8% agar, 1.5% sucrose) for 10 days. The rooted seedlings were then transferred to a mixture of compost and perlite (1:1) and watered very carefully to avoid waterlogging.

3.2.2 Effect Of Antibiotics on Shoot Regeneration
The effects of several antibiotics (carbenicillin, cefotaxime, vancomycin all at 500 mg/l and augmentin at 400 mg/l) on shoot regeneration from 4-8 dpg cotyledons was tested. Cotyledon explants were regenerated on MS medium containing 1 mg/l BAP and 0.1 mg/l NAA supplemented with antibiotics and
Table 3.4 Transformation efficiency (%) in the growth of transgenic callus and transformed shoots from 4 days old cotyledon explants of *Arabidopsis* "C24" in the presence of 50 mg/l kanamycin and augmentin or vancomycin using C58C1 (pGV3850)/pBI121. Medium: MS containing 0.1 mg/l NAA and 1.0 mg/l BAP

<table>
<thead>
<tr>
<th>Antibiotic (mg/l)</th>
<th>Callus</th>
<th>Shoot*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin (500)</td>
<td>84</td>
<td>66</td>
</tr>
<tr>
<td>Augmentin (400)</td>
<td>86</td>
<td>68</td>
</tr>
</tbody>
</table>

Total explants/treatment = 175. Data was recorded 6 weeks after inoculation. *An average of 4 transformed shoots per cultured explant were produced
without antibiotics as a control. After 4 weeks in culture, the number of shoots per explant was similar in vancomycin and augmentin plates and control plates but carbenicillin and cefotaxime gave much lower shoot regeneration (data not shown). It was obvious that cefotaxime and carbenicillin inhibited shoot regeneration whilst shoot production was not inhibited in the presence of vancomycin or augmentin. These antibiotics (vancomycin and augmentin) were added to regeneration and selection medium after co-cultivation in order to kill *Agrobacterium* strains. It was important to use antibiotics which would not inhibit shoot regeneration *in vitro*. Both antibiotics killed *Agrobacterium* successfully without affecting shoot regeneration (Table 3.4). The effects of kanamycin, which was required to select transformed shoots on selection medium was studied at various concentrations (25, 50, 75, 100 mg/l). As shown in Table 3.6, 50 mg/l kanamycin appeared optimum for the selection of kanamycin resistant shoots *in vitro*. At this concentration, all the shoots regenerated from wild-type *A. thaliana* explants were bleached. Higher concentrations (75 and 100 mg/l) completely inhibited shoot regeneration, and 25 mg/l kanamycin allowed the growth of green shoots from control explants.

3.2.3 Transformation of Cotyledon Explants
Cotyledon explants from 4-8 days dpg seeds were used for transformation as these were easier to work with than immature cotyledons. The majority of transformation experiments were carried out on “C24” and are these reported here; however, preliminary experiments suggested that similar results may be obtained for “Landsberg erecta”. Two disarmed *Agrobacterium* strains CS8C1 (pGV3850)/pBI121 and LBA4404 (pAL4404)/pBin19 were used in the transformation experiments. Both binary plasmids (pBI121 and pBin19) carry a neomycin phosphotransferase II (NPT-II) gene driven by the NOS promoter which confers resistance to kanamycin, whilst pBI121 also contains a GUS (β-glucuronidase) gene driven by the CaMV 35S promoter. Cotyledons were either inoculated immediately after dissection or following preculture in regeneration
medium for 2 days. Precultured cotyledons exhibited less growth of transformed callus and shoots. This may have resulted from the lack of a fresh cut surface or less physical damage on the explants for Agrobacterium infection. The nearer the cut was made to the end of the cotyledon proximal to the main axis the higher was the production of transformed shoot growth. This shows that the origin of the tissue used for regeneration is important. It also suggests that a polar phenomenon affecting morphogenesis exists in Arabidopsis cotyledons.

Cotyledon explants that had an attached axillary or apical meristem from the seedling developed axillary shoots within 3-4 days on selection medium (i.e. containing 50 mg/l kanamycin and 400 mg/l augmentin or 500 mg/l vancomycin) which bleached within two weeks making them easy to identify and remove. Green transformed callus started to appear on selection medium within 6-8 days (Figure 3.2) and as many as 68% of explants produced at least one transformed shoot within 4 weeks (Table 3.4). An average of 3-4 transformed shoots per explant was obtained (Figure 3.2). Transformed shoots grew normally and produced fertile flowers and set seeds in vitro or upon transfer to soil.

Agrobacterium strain LBA4404 was not efficient at producing transformants and was eliminated from these studies after a few initial experiments. Agrobacterium strain pGV3850 is a nopaline-type plasmid while pAL4404 is an octopine-type plasmid strain; it is probable that the vir system of the nopaline-type plasmid strain is more efficient for transformation of Arabidopsis cotyledon explants. The first transformed shoots appeared 2-3 weeks after inoculation. Various dilutions of Agrobacterium (i.e. 1/20, 1/50, 1/100) and different periods of inoculation (i.e. 3-180 minutes) were used and a 1/50 dilution for 3 minutes was chosen as a satisfactory inoculation treatment. Higher concentrations of Agrobacterium and longer inoculation periods caused bacterial overgrowth during co-cultivation. In contrast, lower dilutions and shorter inoculation periods proved insufficient to infect the explants. All explants were blotted on sterile filter paper prior to co-cultivation for periods of 2-7 days. Co-cultivation period exceeding four days resulted in overgrowth of explants by
Figure 3.2
Transformation of *A. thaliana* ecotype "C24" with pGV3850/pBI121 which carries a NPT-II gene for kanamycin selection and a GUS gene for screening of transformed plants.

A. Transformed callus formation on MS medium containing 1.0 mg/l BAP and 0.1 mg/l NAA supplemented with 50 mg/l kanamycin (after one week in culture). Bar= 1 cm

B. Transformed shoot regeneration on same medium as above (after four weeks in culture). Bar= 1 cm

C. Histochemical GUS staining of leaves from transgenic plants and wild-type. Leaves taken from kanamycin resistant plants and wild-type plant were incubated at 37 °C in 1-2 ml of X-Gluc (1 mg/ml in 50 mM NaPO₄, pH 7.0) overnight. They were then rinsed in 70% ethanol. Bar=0.25 cm
Agrobacterium causing tissue death. 2-3 days appeared optimal since this allowed some bacterial proliferation at the edges of the explants, but did not allow overgrowth. Vancomycin and augmentin were both satisfactory antibiotics for the removal of Agrobacterium during regeneration from cotyledons (see section 3.2.2); however augmentin was more successful in controlling Agrobacterium after co-cultivation (Table 3.4). Therefore augmentin was used in all transformation experiments reported here. Explants were subcultured to new selection medium containing vancomycin or augmentin twice for two weeks each. Later subcultures were made on medium containing kanamycin only. The absence of Agrobacterium contamination during the later subcultures (without augmentin) demonstrated that transgenic shoots were free from Agrobacterium which is important for GUS assays since Agrobacterium contains endogenous GUS activity.

3.2.4 Histochemical GUS Activity Assay of Transformed Shoots
The presence of GUS activity in kanamycin resistant shoots and callus growing on selection media was confirmed using a histochemical assay since the binary plasmid pBI121 carries a GUS gene driven by the CaMV 35S promoter. CaMV 35S-GUS gene expression was observed in leaves, shoots, stem and flowers of all regenerants grown in selection media (Figure 3.2). GUS expression was stronger in the most actively growing regions of the plants and in young vascular tissues. A total of 22 kanamycin resistant plants were subjected to the histochemical GUS assay and all expressed GUS activity indicating the complete absence of escapes in this population (Table 3.5).

3.2.5 Southern Blot Hybridization
Southern blot hybridization confirmed the integration of at least one copy of the T-DNA into the nuclear genome of each transformed plants (Figure 3.3). Transformed plants were maintained in selection medium containing 50 mg/l kanamycin and no augmentin for at least three months prior to Southern blot
Table 3.5 Number of histochemical GUS stained Arabidopsis plants

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of kanR plants selected randomly*</td>
<td>22</td>
</tr>
<tr>
<td>Number of GUS (+) plants**</td>
<td>22</td>
</tr>
<tr>
<td>Percentage of GUS (+) plants</td>
<td>100</td>
</tr>
</tbody>
</table>

* Green and normal looking plants grown on 50 mg/l kanamycin. ** Records were taken 4 h after incubation with x-gluc.

Table 3.6 Segregation of the kanamycin resistance phenotype in first selfed generation (t1) of transformed Arabidopsis "C24" plants

<table>
<thead>
<tr>
<th>Samples</th>
<th>Sown seeds</th>
<th>Germinated seeds</th>
<th>KanR</th>
<th>KanS</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5</td>
<td>78</td>
<td>75</td>
<td>58 (77%)</td>
<td>17</td>
</tr>
<tr>
<td>A7</td>
<td>45</td>
<td>45</td>
<td>45 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>A10</td>
<td>42</td>
<td>42</td>
<td>32 (76%)</td>
<td>10</td>
</tr>
<tr>
<td>A11</td>
<td>63</td>
<td>60</td>
<td>44 (73%)</td>
<td>16</td>
</tr>
<tr>
<td>A26</td>
<td>67</td>
<td>65</td>
<td>51 (78%)</td>
<td>14</td>
</tr>
<tr>
<td>A30</td>
<td>40</td>
<td>40</td>
<td>29 (73%)</td>
<td>10</td>
</tr>
</tbody>
</table>

Seeds were germinated in vitro on MS medium supplemented with 50 mg/l kanamycin and no hormones.
Figure 3.3
Southern blot analysis of DNA from transformed Arabidopsis plants. Blots were hybridized with a 1.4 kb fragment of NPT-II gene. Lanes: 1) pBI121, PstI; 2) k-marker; 3) GUS (+) plant A11, PstI; 4) GUS (+) plant A11, HindIII; 5) GUS (+) plant A11, KpnI; 6) Wild-type plant, PstI; 7) GUS (+) plant A26, PstI; 8) GUS (+) plant A26, HindIII; 9) GUS (+) plant A26, KpnI. The arrow shows the position of the 1.4 kb PstI fragment of NPT-II gene.
Two transformed plants (A11 and A26) were selected randomly from kanamycin resistant / GUS (+) plants for Southern blot hybridization.

Digestion of DNA with \( PstI \) releases a single internal T-DNA fragment of 1.4 kb (NPT-II gene) (Figure 3.3, lanes 1, 3, 7). Digestion with \( HindIII \), which has single restriction site in the T-DNA, confirmed integration of T-DNA into plant genome (Figure 3.3, lanes 4, 8). \( KpnI \), which has no restriction site in the T-DNA, failed to give a band (Figure 3.3, lanes 5 and 9). \( PsfI \) digested GUS (+) plants and pBI121 plasmid DNA revealed a 1.4 kb internal fragment of the NPT-II gene (Figure 3.3, lanes 1, 3, 7). No hybridization was observed from wild-type \textit{Arabidopsis} plants (Figure 3.3, lane 6).

### 3.2.6 Inheritance of Kanamycin Resistance

Seed from six selfed transformed plants was germinated on MS medium without hormones and containing 50 mg/l kanamycin. After 8 days, seedlings were scored for resistance to kanamycin by observing the bleaching of the plants. This bleaching is characteristic of kanamycin sensitivity since seeds of untransformed plants can germinate on this concentration of kanamycin. Control (wild-type) seedlings bleached in one week, whilst a proportion of seedlings from transformed plants developed and maintained a normal green colour. The germination of seeds from transgenic plants on solid MS medium containing 50 mg/l kanamycin showed that the transmission of the transgene to progeny was in a Mendelian fashion (Table 3.6). Of the six lines tested, five lines demonstrated a 3:1 segregation of resistance to sensitivity within the selfed progeny. This indicated the presence of a single copy of the T-DNA. One line, A7, failed to segregate kanamycin sensitive progeny indicating the presence of multiple T-DNA insertions.
3.3 Discussion

3.3.1 Adventitious Shoot Regeneration Capacity of Immature Cotyledons

The regeneration and transformation potential of *A. thaliana* immature cotyledons were investigated using two ecotypes, "Landsberg erecta" and "C24". Immature cotyledon explants were taken from seed pods at various stages of maturity. Both ecotypes produced similar responses but shoot regeneration was preceded by anthocyanin accumulation in the callus of "Landsberg". Shoots were initiated in about 10 days via callus formation. This relatively rapid shoot regeneration may result in the induction of less somaclonal variation since minimizing the callusing period is expected to reduce somaclonal variation (Larkin and Scowcroft, 1981; Marton and Browse, 1991). This is very important for genetic transformation which requires genetically stable transgenic plants. A very high frequency of both callus formation (up to 100%) and shoot regeneration (95%) was obtained using MS medium supplemented with 0.1 mg/l NAA and 1.0 mg/l BAP. Patton and Meinke (1988), obtained much lower (30-60%) shoot regeneration from cotyledon explants of the *A. thaliana* ecotype Columbia. Shoot regeneration was also high on a wide range of media. A higher concentration of BAP (4 mg/l) caused vitrification and reduced shoot elongation in cultured immature cotyledon explants. These abnormalities are reportedly associated with high concentrations of BAP in shoot regeneration medium (Barghchi and Alderson, 1989).

Cotyledons from *in vitro* germinated seeds were also used for shoot regeneration. The results from these experiments were in agreement with the data on shoot regeneration for "Columbia" ecotype reported by Patton and Meinke (1988), although a higher shoot regeneration frequency was achieved in the present study. This could be due to the ecotype effect or other cultural conditions such as inoculation temperature or reduced stress (dehydration) during explant preparation for culture.
3.3.2 Agrobacterium-Mediated Transformation of Cotyledon Explants

Agrobacterium-mediated transformation was carried out using cotyledon explants from 4-8 day old germinated seeds since these were much easier to handle than immature cotyledons. Although both ecotypes showed similar responses, the majority of transformation experiments were carried out on "C24". Preculturing for 2 days prior to inoculation reduced the transformation efficiency which could be due to the lack of fresh cut surface or the repair of physical damage on the explants, both of which might reduce the efficiency of Agrobacterium infection. The nearer the cut was made to the end of the cotyledon proximal to the main axis the higher was the extent of transformed shoot growth. Similar observations were made in apple (Kouider et al., 1984) and soybean (Mante et al., 1989). Vancomycin and augmentin showed no inhibitory effects on regeneration whilst cefotaxime and carbenicillin both inhibited regeneration. Valvekens et al. (1988) also reported that cefotaxime and carbenicillin had inhibitory effects on regeneration, but vancomycin did not inhibit regeneration from root explants. In the present study, transformed shoots, which appeared green, were produced in 4 weeks at a frequency of 68% and at an average of 3-4 transformed shoots per explant. This transformation procedure produced transformed shoots at a higher frequency and a more rapidly than previous reports (Lloyd et al., 1986; An et al., 1986; Zhang and Somerville, 1987; Feldman and Marks, 1987; Valvekens et al., 1988).

Valvekens et al. (1988) reported that the roots of Arabidopsis represented a good source of explants from which to produce transgenic plants via A. tumefaciens-mediated transformation. Although this method (root) is used most routinely, the present method (cotyledon) may have some important advantages over the root method. Firstly, the preparation of explants is more straightforward and rapid (it takes 3-4 days with cotyledon, 3-4 weeks with root). Secondly, cotyledons produced a higher frequency of transformed plants within 4 weeks, but it took at least three months to regenerate plants from root explants. This
high efficiency of transformed shoot production could be reduced dramatically if cotyledon explants dehydrated or wilted during explant preparation or if cotyledons were cut further way from the proximal end. The callus phase is also very short, reducing the risk of somaclonal variation. This provides a transformation system which allows the routine use of *Arabidopsis* transformation in plant molecular genetics and developmental biology. Therefore, sense and antisense RNA constructs could be tested using this transformation method before transforming *B. napus*.

3.4 Summary

Procedures were developed for rapid and prolific adventitious shoot regeneration of *A. thaliana* (L.) Heynh ecotypes "landsberg erecta" and "C24" from cotyledon explants at 90-100% efficiency. Immature cotyledons had the highest shoot regeneration efficiency. Prolific regeneration was achieved in Murashige and Skoog medium supplemented with 0.1-0.4 mg/l naphthaleneacetic acid and 1.0 mg/l 6-benzylaminopurine within 2 weeks. The regenerated plants had a normal phenotype and produced fertile flowers and set seeds. The above regeneration protocol was used to develop a transformation method utilising the disarmed *A. tumefaciens* strain pGV3850/pBI121 and kanamycin selection. Transgenic shoots were produced within 2-3 weeks after inoculation. Transformation of shoots was confirmed by GUS histochemical assay, as well as Southern blot hybridization.
CHAPTER 4

REGENERATION AND AGROBACTERIUM-MEDIATED TRANSFORMATION OF B. NAPUS

4.1 Introduction

The principal purpose of investigating ways of improving Brassica napus gene transfer techniques was to test the effect of A1 and A9 antisense RNA genes. However, B. napus is also one of the world's most important sources of vegetable oil and protein meal and therefore has become the object of extensive tissue culture studies and genetic engineering techniques have been applied to B. napus to introduce new genes (Knutzon et al., 1992). Generally, efficient transformation methods require good regeneration capacity. To date, organogenesis has been achieved in a variety of explants such as stem sections (Stringam 1977; Pua et al., 1991), stem thin cell layers (Klimanszewska and Keller 1985), leaf discs (Dunwell 1981), roots (Lazzeri and Dunwell 1984; Sharma and Thorpe 1989), cotyledons (Narasimhulu and Chopra 1988;
Moloney et al., 1989), hypocotyls (Dieter et al., 1982; Radke et al., 1988) and protoplast cultures (Xu et al., 1982; Barsby et al., 1986; Loudon et al., 1989). Embryogenesis has been reported in hypocotyl tissues (Kohlenbach et al., 1982; Loh and Ingram 1982), mesophyll protoplast cultures (Li and Kohlenbach, 1982), and anther and pollen cultures (Keller and Armstrong 1978; Lichter 1982). Despite these reports, the frequency of shoot regeneration and the number of regenerated plants remain low and the process is usually protracted. An additional problem is that the long period of in vitro incubation during shoot regeneration may increase both the possibility of somaclonal variation and the risk of changes in ploidy level (De Block, 1988; Marton and Browse, 1991). This is problematic for in vitro micropropagation experiments.

Agrobacterium-mediated transformation of B. napus has been achieved using various explant types: stem segments (Fry et al., 1987), thin cell layers (Charest et al., 1988; Misra, 1990), hypocotyls (Radke et al., 1988; De Block et al., 1989), cotyledonary explants (Moloney et al., 1989), inflorescence stalks (Boulter et al., 1990), and microspore-derived embryos (Swanson and Erickson, 1989). However, transformation efficiencies were still poor. Despite several authors reporting higher than 20% of transformed shoot frequency (Fry et al., 1987; De Block et al., 1989; Moloney et al., 1989), their methods could not be used routinely by other groups. The remaining transformation methods achieved transformation frequencies of between 0.5 and 3%. Therefore, we attempted to improve both the shoot regeneration capacity and Agrobacterium-mediated transformation efficiency of B. napus. For this aim, various explant types and plant growth regulators were investigated in vitro, together with various Agrobacterium tumefaciens strains, in order to identify the most appropriate strain for transformation of B. napus.
4.2 Results

4.2.1 Effect of Light and Temperature on Regeneration of Cotyledonary Explants

2/3 distal cotyledon explants excised from 5-day old seedlings (Figure 4.1) were used to investigate the effect of light and temperature on regeneration (Table 4.1). 2/3 distal cotyledon explants were obtained by removing the basal end of the lamina as shown in Figure 4.2. Cotyledon explants were placed onto MS medium supplemented with 4 mg/l BAP and 0.5 mg/l NAA and cultured in various temperature and light (40 μEm⁻²s⁻¹) regimes: 26°C and light; 26°C and dark; 20°C and light; 20°C and dark. Of the treatments, 26°C and light gave the highest (70%) shoot regeneration followed by 20°C and light (20%). Two dark treatments did not induce shoot regeneration (Table 4.1).

4.2.2 Efficient Shoot Regeneration from 2/3 Distal Cotyledon Explants

2/3 distal cotyledon explants were excised from 5-day old seedlings that were grown in sterile conditions. The basal end of the lamina was removed from each cotyledon and the cut surface of the explants was dipped into MS medium supplemented with BAP and NAA at a density of 5 explants per 9 cm plate. They were cultured at 26°C and in a 16 hour photoperiod. A high frequency of adventitious shoot regeneration was achieved from 2/3 distal cotyledonary explants (Table 4.2). Callus formation was apparent within ten days, and in four weeks adventitious shoots were regenerated. MS medium supplemented with 2, 4 and 8 mg/l BAP were tested with 0.5 mg/l NAA or without NAA. The highest frequency of shoot regeneration (60%) was obtained from 2 mg/l BAP + 0.5 mg/l NAA followed by 4 mg/l BAP + 0.5 mg/l NAA (65%) (Table 4.2, Figure 4.3). However, 4 mg/l BAP + 0.5 mg/l NAA gave a higher number of shoots per explant compared to medium with 2 mg/l BAP + 0.5 mg/l NAA (Table 4.2). Conversely, increasing the BAP concentration to 8 mg/l 0.5 mg/l NAA reduced shoot regeneration to 40% (Figure 4.3). The highest frequency of callus
Figure 4.1
A. 5-day old *B. napus* seedlings grown on MS0 medium in a sterile condition. Bar= 1 cm

B. Regeneration stages from a cotyledon with petiole explant; from left to right, initial cotyledonary explant after one week and 4 weeks in culture, respectively. Bar= 1 cm

C. High frequency adventitious shoot regeneration on MS medium containing 4 mg/l BAP, 2 mg/l kinetin and 0.1 mg/l NAA (after four weeks in culture). Bar= 1 cm
Table 4.1 Effect of light and temperature on shoot regeneration.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Frequency of Shoot Growth (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light, 26 °C</td>
<td>70</td>
</tr>
<tr>
<td>Light, 20 °C</td>
<td>20</td>
</tr>
<tr>
<td>Dark, 26 °C</td>
<td>0</td>
</tr>
<tr>
<td>Dark, 20 °C</td>
<td>0</td>
</tr>
</tbody>
</table>

Data scored after 4 weeks in culture: 30 expliants per treatment.

Table 4.2 Effect of BAP and NAA concentrations on regeneration of 2/3 distal cotyledons.

<table>
<thead>
<tr>
<th>Plant growth regulators (mg/l)</th>
<th>Callus(^1) (%)</th>
<th>Shoot(^1) (%)</th>
<th>Number of(^2) shoot/per explant</th>
<th>Root(^1) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP  0.0  NAA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4  0.0  0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8  0.0  0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2  0.5  20</td>
<td>80</td>
<td>2.4±1.0</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>4  0.5  55</td>
<td>65</td>
<td>2.9±1.5</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>8  0.5  25</td>
<td>40</td>
<td>1.7±0.8</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Frequency of regeneration (percentage of regenerated explants). \(^2\) ± represents standard error of mean. Data scored after 4 weeks in culture: 40 explants per treatment.
Figure 4.2
The two types of cotyledonary explants used for *in vitro* regeneration.

A. 2/3 distal cotyledon explant excised from 5-day old seedling. The basal end (1/3) of the lamina was removed.

B. Cotyledon with 2-3 mm long petiole explant excised from 5-day old seedling.
formation was also obtained with 4 mg/l BAP + 0.5 mg/l NAA (Table 4.2). Without NAA, no organogenesis occurred and all the explants died within one month. These results showed that NAA (an auxin) is crucial for regeneration from 2/3 distal cotyledons. When 0.5 mg/l NAA was added to the medium, organogenesis was initiated. 4 mg/l BAP + 0.5 mg/l NAA produced good callus formation (55%) and shoot regeneration (65%) which are very important for Agrobacterium-mediated transformation. Organogenesis was initiated at the cut surface of the explants. Adventitious shoots were initiated through callus formation or directly from the cut surface.

4.2.3 High Frequency of Shoot Regeneration from Cotyledon with Petiole Explants

Preliminary experiments showed that cotyledon with petiole explants (Figure 4.1) were superior to cotyledons without petiole. Seedling age and culture density were also investigated using cotyledon with petiole explants. 5 explants per 9 cm plate taken from 5-day old seedlings gave the best regeneration capacity (60%). A higher culture density (10 explants per plate) reduced shoot regeneration (25%).

Cotyledonary explants with 2-3 mm long petioles also showed very good shoot regeneration capacity. Cotyledons with petioles taken from 5-day old sterile seedlings were embedded in the solid MS medium containing plant growth regulators. 5 explants per 9 cm plate were plated. BAP and kinetin (cytokinins), and NAA and IAA (auxins) were examined in various combinations (Table 4.3). Adventitious shoots were produced via callus formation at the cut surface within 4 weeks (Figure 4.1). MS medium supplemented with 4 mg/l BAP + 2 mg/l kinetin + 0.10 mg/l NAA gave the highest frequency of callus formation (100%), shoot regeneration (90%) and average number of shoots per explant (2.9) (Table 4.3, Figure 4.1). In contrast, MSO medium alone failed to induce callus or shoot regeneration, but roots were formed on 50% of explants. 4 mg/l BAP with 0.5 mg/l NAA or 0.5 mg/l IAA induced a similar shoot regeneration
Figure 4.3

Effect of BAP and NAA concentrations on shoot regeneration from 2/3 distal cotyledons. 2/3 distal cotyledon explants excised from 5-day old seedlings were inoculated into MS medium containing 2, 4 and 8 mg/l BAP with 0.5 mg/l NAA and without NAA. Data scored after four weeks in culture (40 explants per treatment).
BAP concentrations

0 mg/l NAA
0.5 mg/l NAA

Shoot growth (%)
Table 4.3 Effect of various cytokinin and auxin combinations on regeneration from cotyledon with petiole explants

<table>
<thead>
<tr>
<th>Plant growth regulators (mg/l)</th>
<th>Callus$^1$ (%)</th>
<th>shoot$^1$ (%)</th>
<th>Number of$^2$ shoot per explant</th>
<th>Root$^1$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP</td>
<td>Kin</td>
<td>NAA</td>
<td>IAA</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.50</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0.25</td>
<td>-</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0.10</td>
<td>-</td>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0.10</td>
<td>-</td>
<td>0.50</td>
<td>0</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>0.25</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>0.50</td>
<td>0</td>
<td>40</td>
</tr>
</tbody>
</table>

$^1$ Frequency of regeneration (percentage of regenerated explants). $^2$ ± represents standard error of mean. Data scored after 4 weeks in culture: 25 explants per treatment.
frequency (70%), but 4 mg/l BAP + 0.5 mg/l NAA produced more callus formation and root regeneration than 4 mg/l BAP + 0.5 mg/l IAA (Table 4.3).

These results showed that NAA induced callus and root regeneration more efficiently than IAA. The lowest shoot regeneration frequencies were obtained from 4 mg/l BAP + 4 mg/l kinetin + 0.25 mg/l NAA (20%) and 4 mg/l BAP + 0.25 mg/l IAA (30%) (Table 4.3). Except for MS0 and media containing IAA, all other treatments showed very high frequency of root regeneration (100%). In these cases organogenesis was initiated at the cut surface of the cotyledonal explants. 4 mg/l BAP + 2 mg/l kinetin + 0.10 mg/l NAA produced adventitious shoots via callus formation (Figure 4.1).

4.2.4 Callus Formation from Immature Cotyledons

Small immature cotyledon explants swelled rapidly during the first 2-3 days on culture medium. Callus formation was initiated at the cut surface of the immature cotyledonal explants. Callus proliferation was obtained at frequencies varying from 0 to 100%. The results are summarised in Table 4.4. The highest frequency of callus formation (100%) was obtained using medium 3 (10 mg/l NAA), medium 5 (40 mg/l NAA), and medium 5 in a 2-step regime - a preculture period of ten days on medium 5 followed by culture on MS0. This latter treatment together with continuous culture on medium 3 induced the formation of some embryogenic callus. However, continuous culture on medium 5, ie without transfer to MS0, induced the formation of pale and nonembryogenic callus which died during early stages of culture. Medium 10 (4 mg/l BAP and 1 mg/l NAA) failed to give callus formation; and media 6, 7 and 9, which contained BAP, produced very low frequencies of callus formation (12.5%). The 2-step regime in which explants were precultured on MS0 media containing 5, 10 and 20 mg/l NAA (media 2, 3 and 4, respectively) and then transferred to MS0 reduced the frequency of callus formation compared to control explants maintained on the original media. Medium 2 (5 mg/l NAA) induced the highest frequency of embryogenic callus formation, followed by medium 3 (10 mg/l NAA).
Table 4.4 Effect of NAA and BAP on regeneration of *B. napus* immature cotyledon explants

<table>
<thead>
<tr>
<th>Medium No.</th>
<th>Growth (mg/l)</th>
<th>Callus 1 (%)</th>
<th>Shoot 1 (%)</th>
<th>Number of shoots per explant</th>
<th>Root 1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA</td>
<td>BAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>33.0</td>
<td>67.0</td>
<td>4.6 ± 0.8</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>0</td>
<td>60.0</td>
<td>100.0</td>
<td>3.2 ± 0.9</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>20.0</td>
<td>60.0</td>
<td>2.8 ± 1.3</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>0</td>
<td>100.0</td>
<td>100.0</td>
<td>7.3 ± 1.2</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>0^3</td>
<td>50.0</td>
<td>75.0</td>
<td>3.7 ± 1.6</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>0</td>
<td>57.0</td>
<td>71.5</td>
<td>2.4 ± 2.3</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>0^3</td>
<td>30.0</td>
<td>70.0</td>
<td>2.7 ± 1.5</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>0</td>
<td>100.0</td>
<td>40.0</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>0^3</td>
<td>100.0</td>
<td>80.0</td>
<td>3.2 ± 2.0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0.25</td>
<td>12.5</td>
<td>75.0</td>
<td>6.8 ± 5.2</td>
</tr>
<tr>
<td>7</td>
<td>0.25</td>
<td>1</td>
<td>12.5</td>
<td>50.0</td>
<td>4.0 ± 3.3</td>
</tr>
<tr>
<td>8</td>
<td>0.25</td>
<td>4</td>
<td>25.0</td>
<td>62.5</td>
<td>4.1 ± 2.0</td>
</tr>
<tr>
<td>9</td>
<td>0.50</td>
<td>4</td>
<td>12.5</td>
<td>75.0</td>
<td>2.3 ± 1.2</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>4</td>
<td>0.0</td>
<td>62.5</td>
<td>3.9 ± 1.4</td>
</tr>
</tbody>
</table>

1 Frequency of regeneration (percentage of regenerated explant). 2 ± represents standard error of mean. 3 Denotes 2-step culture - transfer to MSO medium after 10 days culture. Data scored after 4 weeks in culture: 40 explants per treatment.
Figure 4.4
Effect of NAA concentration on organogenesis of *B. napus* immature cotyledon explants. Treatments involved continuous or two-step (10 days on hormone-containing medium, followed by transfer to MS0) culture on medium 2 (5 mg/l NAA), 3 (10 mg/l NAA), 4 (20 mg/l NAA) or 5 (40 mg/l NAA). * denotes 2-step culture regime.
Callus
Shoot
Root

Medium numbers as in Table 3
When compared to MS0 medium (medium 1), NAA increased the frequency of both callus and embryogenic callus formation. Conversely, the incorporation of BAP into any of the culture media had the effect of dramatically reducing the frequency of callus formation.

4.2.5 Organogenesis from Immature Cotyledons

Four different modes of shoot regeneration were obtained from immature cotyledon explants. The first was direct adventitious shoot regeneration. Where this occurred, the petioles started to split within two weeks and shoot primordia appeared inside the petiole after a further week of culture. This regeneration system apparently did not involve the formation of a callus intermediate so that shoots originated directly from the subepidermal tissues of the cotyledonary petiole. In the second regeneration system, shoot proliferation occurred through a callus phase which was initiated at the cut end of the explants within 2 weeks. The third mode involved direct somatic embryogenesis and the fourth somatic embryogenesis via callus formation.

The immature cotyledonary explants showed rapid and multiple shoot regeneration. The highest number of shoots per explant (7.3) was obtained within four weeks by continuous culture on medium 3 (10 mg/l NAA); medium 6 (0.25 mg/l BAP) also gave a high number of shoots (6.8) (Table 4.4). However, medium 3 induced a mixture of adventitious shoots and somatic embryos whilst medium 6 induced only direct shoot regeneration. Both treatments also induced a number of shoot primordia. Explants subjected to continuous culture on medium 5 exhibited the lowest frequency of shoot regeneration (1.00 shoot per explant) (Table 4.4).

MS medium without growth regulators (MS0, medium 1) induced the regeneration of a relatively high number of shoots (4.60). However, 2-step treatments in which the initial phase of culture involved low NAA concentrations (media 2 and 3), had the effect of depressing the extent of shoot regeneration upon transfer to MS0. Conversely, where the initial phase involved the higher
NAA concentrations (media 4 and 5), the number of shoots was increased by transfer to MSO. The highest frequencies of shoot regeneration (100%) were obtained by continuous culture in media 2 (5 mg/l NAA) and 3 (10 mg/l NAA) (Table 4.4).

The adventitious shoots induced by the treatments outlined above were initiated either directly from subepidermal tissues or embryoids formed on the surface of the petiole, or indirectly through embryogenic callus. However, medium containing BAP produced shoots only via direct organogenesis. Roots also initiated on the cut surface of the explants. The highest frequency of root regeneration (90%) was obtained by a 2-step regime with medium 4 (Table 4.4) indicating that root regeneration was increased dramatically by transfer to MSO after an initial period on media containing NAA (Figure 4.4). Medium 6 (0.25 mg/l BAP) did not induce root formation, but medium 7 (1 mg/l BAP and 0.25 mg/l NAA) gave high frequency of root regeneration.

4.2.6 Somatic Embryogenesis from Immature Cotyledons

To investigate somatic embryogenesis in detail, immature cotyledon explants were cultured on medium 2 (5 mg/l NAA) which produced the highest frequency of somatic embryogenesis. Two types of somatic embryogenesis were obtained from immature cotyledonal explants (Figure 4.5A and B). They were direct somatic embryogenesis and somatic embryogenesis via callus formation. Direct somatic embryos initiated from cotyledonary margins within 10 days. They were green, tubular in shape and attached to the explant in groups of 3-5 (Figure 4.5A and B). Dissection of regenerated explants showed that embryogenesis occurred within the epidermal tissues in the absence of callus proliferation. Within 2 weeks, cotyledons opened at the distal end of the tubular embryoids and some produced roots (Figure 4.5B). They were then separated from the explants and transferred to MS0 medium to induce root regeneration. The embryoids produced strong main roots and several leaves within 5-6 days (Figure 4.5C) and were then transferred to soil.
Figure 4.5

Somatic embryogenesis on medium 2 (5 mg/l NAA) from immature cotyledons of *B. napus*. Bar= 0.5 cm

A. Early stage of direct somatic embryogenesis initiated from epidermal tissues within 10 days. Bar= 0.5 cm

B. Tubular shaped direct embryos after 2 weeks in culture. Bar= 0.5 cm

C. A direct embryo separated from the explant and rooted on MS0 medium (after 3 weeks in culture). Bar= 0.5 cm

D. Embryogenic callus initiated at the cut surface of the explant within 2 weeks. Bar= 0.5 cm

E. Indirect cotyledonary-stage embryos after 3 weeks in culture. Bar= 0.5 cm

F. Growing cotyledons of somatic embryos within 4 weeks. Bar= 0.5 cm
The second type of somatic embryogenesis was indirect embryogenesis and involved a callus phase (Figure 4.5). Embryogenic callus, which was characteristically shiny and harder than normal callus (Figure 4.5D), initiated at the cut surface of the explants within two weeks. Cotyledons of somatic embryos appeared via callus stage within 3 weeks (Figure 4.5E and F). An average of 5-6 embryos in different stages developed from one explant. The two types of somatic embryogenesis exhibited different pattern of development. Direct embryos were tubular and appeared within 10 days whilst embryos via a callus stage appeared within 3 weeks and consisted of recognizable cotyledons from the outset. It was also noted that the two modes of embryogenesis were associated with different parts of the explant: where the cut end was cotyledonary petiole, embryogenic callus formation occurred, but if the cut end was distal cotyledon, direct embryogenesis was obtained.

Immature cotyledons gave high frequency of somatic embryogenesis. Out of 140 explants, 116 (83%) produced at least 3-4 somatic embryos. 40% of the explants showed direct somatic embryogenesis and 43% produced somatic embryos via callus formation.

### 4.2.7 Effect of Explant Type on Shoot Regeneration from Immature Cotyledons

Separate experiments showed that the petiole was the most regenerable tissue of the cotyledonary explant. Immature cotyledons were dissected with and without a petiole and cultured on MS0 medium containing 4 mg/l BAP + 0.5 mg/l NAA. The treatment of explants in this way induced 80% shoot regeneration at 2.1 shoots per explant (Table 4.5). However, when the petiole was removed, the frequency of shoot regeneration was reduced to 30%, and the number of shoots per explant was reduced to 1.3 (Table 4.5). Therefore, for shoot multiplication, cotyledonary explants should be dissected carefully in order to ensure that the petioles are retained.
Table 4.5  Petiole influence on shoot regeneration

<table>
<thead>
<tr>
<th>Explant Type</th>
<th>Shoot(%)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>No. of Shoots&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature cotyledon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>with petiole</td>
<td>80.0</td>
<td>2.10 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immature cotyledon</td>
<td>30.0</td>
<td>1.30 ± 0.5</td>
</tr>
<tr>
<td>without petiole</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Frequency of shoot regeneration (percentage of regenerated explant)

<sup>2</sup> Number of shoot per explant, ± represents standard error of mean

Medium= MS medium supplemented with 4 mg/l BAP and 0.5 mg/l NAA

Data scored after 4 weeks in culture: 80 explants per treatment.
4.2.8 *Agrobacterium*-Mediated Transformation of *B. napus*

Initially, the concentration of kanamycin required to select transformed plants was investigated using 10, 15, 20, 25 and 30 mg/l kanamycin. It was clear that 10 mg/l kanamycin was insufficient to prevent regeneration of wild-type *B. napus* *in vitro*. Concentrations greater than 20 mg/l halted regeneration completely; but in 15 mg/l kanamycin, 10% of the explants produced bleached shoots. Therefore, 15 and 20 mg/l kanamycin were used to select transformed shoots.

Various wild-type oncogenic *A. tumefaciens* strains were used to identify the most appropriate vir (virulence) system for transformation of *B. napus*. Nopaline strains (C58, T37, A281) and octopine strains (ACH5, A6, A136 NC) were co-cultivated with *B. napus* cotyledonary explants on MS0 medium. Of the oncogenic strains, only the C58 wild-type nopaline strain gave tumours at high frequency (Figure 4.6). For this strain, 20% of the explants produced tumours following C58 infection. Therefore, C58C1(pGV3850)::pBI121 strain, which has the same chromosomal background as C58 was used for transformation of *B. napus*. The binary plasmid pBI121, which carries a NOS-NPTII-NOS gene and confers resistance to kanamycin, and the GUS (β-glucuronidase) gene driven by the CaMV 35S promoter, was introduced into this background. Various experiments were conducted in order to determine the appropriate co-cultivation conditions for this strain. The effect on regeneration of the antibiotics used to kill *Agrobacterium* following co-cultivation were examined; these included carbenicillin (100 mg/l), cefotaximine (250 mg/l) and augmentin (400 mg/l). Augmentin appeared to be very effective against the C58C1(pGV3850)::pBI121 strain after co-cultivation, and apparently had no negative effects on regeneration. The inclusion of silver nitrate (AgNO₃) in the selection/regeneration medium, as indicated by the work of De Block *et al.* (1989), increased shoot regeneration slightly. Silver nitrate has a stimulating effect on shoot regeneration due to inhibition of ethylene action (Purnhauser *et*
Figure 4.6
Transformation of *B. napus* cotyledonary explants.

**A.** Tumour formation on a cotyledon explant of *B. napus* cv. Topaz induced by the wild-type oncogenic *Agrobacterium* strain C58. Bar= 0.5 cm

**B.** Transformation of *B. napus* cotyledonary explants with pGV3850::pBI121 on MS medium+4 mg/l BAP. Top, control plate without co-cultivation; left, transformed shoot regeneration on 20 mg/l kanamycin after three days co-cultivation (one explant with green shoot regenerated via callus); right, bleached shoot regeneration on 20 mg/l kanamycin after three days co-cultivation (Two green cotyledon explants with bleached shoots). Bar= 1 cm
0, 2.5, 5.0 and 10 mg/l silver nitrate were added to the regeneration medium; 2.5 and 5.0 mg/l increased shoot regeneration by 25%, but 10 mg/l reduced this frequency by 50%. Therefore, 5 mg/l silver nitrate was used in the selection medium to improve shoot regeneration.

Various infection methods and inoculation times were evaluated to identify the most appropriate infection regime. In the first method, a 5 ml of an overnight culture of pGV3850::pBI121 was pelleted and resuspended in 10 ml liquid MS0. Cotyledon explants were dipped into the suspension for 5, 10 and 20 seconds. In the second method, an overnight culture was diluted to 1:25 and 1:50 in MS0 and the cotyledon explants were infected for 2 and 15 minutes. The explants were incubated on MS medium containing 4 mg/l BAP and 0.5 mg/l NAA supplemented with antibiotics. In order to score the effectiveness of the various treatments, callus from different treatments was subjected to the histochemical GUS assay and observed under the microscope after dissection. The first and second methods - infection times of 5 seconds and 2 minutes, respectively both with a 1:25 dilution - produced blue spots (transformed cells) in the callus (Figure 4.7). The duration of co-cultivation was also examined and three days appeared optimal since longer than three days caused overgrowth of Agrobacterium around the explants.

The general transformation protocol which was adopted was as follows: cotyledon and petiole explants excised from 5-day old seedlings of B. napus cv Cobra and Topaz were placed on MS medium containing 4 mg/l BAP and 5 mg/l silver nitrate at a density of 10 explants per 9 cm plate. An overnight culture of pGV3850 :: pBI121 (5 ml) was pelleted by centrifugation at 13,000 rpm for 5 minutes. The pellet was then resuspended in 10 ml of MS0 liquid medium (Moloney et al., 1989). The cut surface of the individual excised cotyledons was dipped into this bacterial suspension for 5 seconds then immediately returned to the same plates for co-cultivation. After three days co-cultivation, explants were transferred to regeneration/selection medium supplemented with 20 mg/l kanamycin and 400 mg/l augmentin. Green shoots appeared, via callus
Figure 4.7

Histochemical GUS staining in callus sections and vegetative organs of transformed *B. napus*. Callus sections and vegetative organs taken from kanamycin resistant plants were incubated at 37 °C in 1-2 ml of X-Gluc (1 mg/ml in 50 mM NaPO₄, pH 7.0) overnight. They were then rinsed in 70% ethanol.

A. Callus sections, Bar= 0.25 cm
B. Leaves, Bar= 0.6 cm
C. Stems, Bar= 0.5 cm
D. Root, Bar= 0.5 cm
Figure 4.8
Histochemical GUS staining in various flower organs. Flower buds were fixed in NaPO₄ buffer (pH 7.0) containing 0.1% formaldehyde, 0.1% Triton X-100 and 0.1% β-mercaptoethanol by vacuum infiltration. They were then incubated at 37 °C in 0.001 M X-Gluc overnight. After that buds were fixed in 5% formaldehyde, 5% acetic acid and 20% ethanol for 2 hours. Other flower organs were treated as vegetative organs,

A. Flower buds in a range of bud length (<1mm to 5<mm). Bar= 0.25 cm

B. Petal and sepals, Bar= 0.5 cm

C. Stamens and carpel, Bar= 0.5 cm

D. Seed pod containing seeds, Bar= 0.25 cm
formation, from cultivar Cobra within 4 weeks (Figure 4.6). 30% of the explants produced callus, but only 5% of the explants gave green shoots. A leaf taken from each green shoots was analysed by the histochemical GUS assay and all showed extensive positive blue staining (Figure 4.7). Transformed shoots were transferred to rooting medium. Generally, within 10 days they had formed sufficient root to allow transfer to soil.

In each case the plants grew and set seed normally. Seeds of the transformed plants (T1) were sown onto MS0 medium supplemented with 50 mg/l kanamycin to investigate inheritance of the NPTII gene. 70 T1 seed; (T2 generation) and 50 wild-type B. napus seeds were grown on kanamycin. All the T1 seed germinated to give green seedlings, whilst the wild-type seeds either did not germinate or the resulting seedlings bleached. Since no kanamycin sensitive offspring were identified in the T2 generation, the number of T-DNA loci probably exceeded two. Some of the kanamycin resistant plants were analysed further by histochemical GUS assay. Leaves, stems, roots (Figure 4.7), flower buds, flower (sepal, petal carpel and anther) and seed pods (Figure 4.8) stained blue strongly, but in the anthers only the vascular bundle appeared to express detectable levels of GUS. This result suggested that the activity of the CaMV 35S promoter is restricted to the vascular bundle of anther. Also Fluorimetric GUS assays, which are generally more sensitive than the histochemical assay, were also performed with anthers at various developmental stages as determined by anther length (<1 mm to 5< mm) and the result was shown in Figure 4.9.

Cultivar Topaz did not regenerate any transformed shoot. Although, 50% of the explants produced callus only 3% of the explants gave green shoots. However, these green shoots appeared to be GUS negative (escapes) by histochemical GUS assay.
4.3 Discussion

Efficient shoot regeneration was achieved from cotyledonary explants of *B. napus*. Three different types of cotyledonary explants were investigated in combination with various plant growth regulators. Using cotyledonary explants is much more straightforward than using stem sections, thin cell layer, flower organs or leaf explants, as they are easily grown in sterile condition and are ready for use within 5 days after sowing. Moreover, cotyledonary explants were ideal for *A. tumefaciens* infection since organogenesis occurred through a callus phase at the cut surface. This is an important consideration since a prerequisite for a successful transformation is that the bacterium has direct access to regenerable cells. First, both organogenesis and somatic embryogenesis were obtained and then *A. tumefaciens*-mediated transformation was attempted to improve transformation efficiency.

4.3.1 Regeneration and Transformation of Cotyledonary Explants

The cotyledonary explants showed a high frequency of adventitious shoot regeneration. As reported previously, organogenesis was initiated at the cut surface of the cotyledonary explants of *Brassica* species (Moloney *et al.*, 1989; Sharma *et al.*, 1991; Khehra and Mathias, 1992). The present study used 2/3 distal cotyledon and cotyledon+petiole explants. 2/3 distal cotyledonary explants clearly responded to BAP and NAA. This contrasts with the work of Sharma *et al.*, (1991), who showed that organogenesis from cotyledonary explants of *Brassica juncea* occurred at the petiolar cut end and that in the absence of the petiole regeneration from the lamina was rare. BAP (2, 4, 8 mg/l) without NAA failed to promote regeneration (Table 4.2) and all the explants died within one month. Previous reports also showed that cotyledonary explants from *Brassica* species require both cytokinin and auxin for organogenesis (Narasimhulu and Chopra, 1988; Hachey *et al.*, 1991; Khehra and Mathias, 1992). 2 and 4 mg/l BAP with 0.5 mg/l NAA showed good regeneration capacity but higher BAP concentrations reduced organogenesis (Table 4.2). These
Figure 4.9
The temporal pattern of GUS activity in the anthers of *B. napus* plants transformed with CaMV 35S promoter-GUS. Anther protein was isolated from buds of length <1 mm to >5 mm of transformed *B. napus* plant. Fluorimetric GUS assay was performed using 4-MUG and fluorescence values were standardized relative to the quantity of total protein present in the reaction.
results showed that 2/3 distal cotyledonary explants had a capacity for rapid and high frequency adventitious shoot regeneration. This was considered very useful for *Agrobacterium*-mediated transformation.

Cotyledons with petiole explants gave a very high frequency of adventitious shoot regeneration and callus formation with 4 mg/l BAP + 2 mg/l kinetin + 0.1 mg/l NAA in four weeks (Table 4.3). Two cytokinins (BAP and kinetin) with a very low concentration of NAA produced very good regeneration. To date, no report has mentioned the use of two cytokinins together with cotyledonary explants of *B. napus*. Organogenesis occurred at the cut end of the cotyledonary petiole as previously reported (Narasimhulu and Chopra, 1988; Moloney *et al.*, 1989). Importantly with respect of *Agrobacterium*-mediated transformation, adventitious shoots were initiated through callus formation. Therefore cotyledon-petiole explants were used to transform *B. napus* cv Topaz and Cobra. Initial experiments revealed that 15-20 mg/l kanamycin was effective in selecting against untransformed shoots. Moloney *et al.* (1989) used 15 mg/l kanamycin to select transformed shoots from cotyledonary explants of *B. napus*. The wild-type oncogenic *A. tumefaciens* nopaline-strain C58 was found to be the most effective in tumour-induction on *B. napus* which was in agreement with Charest *et al.* (1989). Holbrook and Miki, (1985) also reported that *B. napus* was readily infected by *A. tumefaciens* nopaline-type strains but not octopine-type strains. *B. napus* has previously been transformed with nopaline-type vectors (Fry *et al.*, 1987; Charest *et al.*, 1987; Radke *et al.*, 1988). C58C1(pGV3850) :: pBl121 which has the same chromosomal background as C58 was used to transform *B. napus* in this work.

2.5-5.0 mg/l AgNO₃ (silver nitrate) increased shoot regeneration in selection/regeneration medium. De Block *et al.* (1989) reported that the use of silver nitrate was a prerequisite for efficient shoot regeneration under selective conditions from *B. napus* and *B. oleracea*. It has been suggested that Ag⁺ blocks ethylene action by binding to the ethylene receptors (Beyer, 1976; Purnhauser *et al.*, 1987).
In the present work, transformed shoots were produced from *B. napus* cv Cobra in 4 weeks. Green shoots grown on 20 mg/l kanamycin were subjected to histochemical analysis and proved to be expressing GUS. Since one of the aims of this work was to drive antisense RNA expression in anther tissues, possibly using the CaMV35S promoter, anthers of transformed plants were examined for GUS. This showed that GUS expression was found only in the vascular bundle of the anther. Plegt and Bino (1989) also reported that CaMV35S promoter-GUS had strong activity in the vascular bundle, but no detectable activity in the tapetum and sporogenous cells. Similarly, Dong et al. (1991) reported that the CaMV 35S promoter was very active in vascular bundles and young tissues of transgenic melon plants. Van der Meer et al. (1992) demonstrated that the CaMV 35S promoter drove GUS expression in the vascular bundle, connectivum and the endothecium of the anther in transgenic petunia plants, but no GUS expression was found in the tapetal cells. These experiments, together the observations reported here, indicate that the CaMV35S promoter is probably transcriptionally inactive within the tapetal cells.

Transformed plants were vegetatively normal and produced normal quantities of seed. These seeds (selfed) were germinated on MS0 medium containing kanamycin to determine the inheritance of the T-DNA. All the seeds appeared to be kanamycin resistant indicating that multiple T-DNA insertion occurred. Moloney et al. (1989) obtained efficient transformation from *B. napus* cv Westar cotyledonary explants using different *A. tumefaciens* strain. Although they achieved a high frequency of transformation in their conditions, the efficiency of transformation still remained low.

### 4.3.2 Organogenesis and Somatic Embryogenesis from Immature Cotyledonary Explants

Immature cotyledon explants from *Brassica napus* showed very high adventitious shoot regeneration and somatic embryogenesis. Immature cotyledons have been cultured successfully for prolific adventitious shoot
regeneration and somatic embryogenesis from other species; soybean (Lazzeri et al., 1985) and chickpea (Shri and Davis, 1992). However, to date the successful utilisation of immature cotyledons from *B. napus* has not been reported. Organogenesis from these explants occurred directly from the subepidermal tissues of cotyledonal petiole and at the cut surface of the explant. Previous reports also indicated that initiation of regeneration from cotyledonal explants of *Brassica* species takes place at the cut end of the petioles (Narasimhulu and Chopra, 1988; Moloney et al., 1989; Hachey et al., 1991; Sharma et al., 1991). However, in contrast, none of the mature cotyledonal explants produced direct shoot regeneration. The present results showed that immature cotyledonal explants underwent a different response than mature cotyledons during *in vitro* culture. Direct organogenesis has been obtained on petiole explants of *B. napus* (Stringam, 1979) and *Beta vulgaris* (Detrez et al., 1988). These authors reported that organogenesis initiated from subepidermal tissues of the petiole explants; this observation was confirmed by the present study where removal of the petiole was found to dramatically reduce shoot regeneration from immature cotyledons of *B. napus* (Table 4.5). Sharma et al. (1991) reported that in the absence of petiole, organogenesis from the lamina was rare and that regeneration from the petioles was also dependent on the presence of lamina for at least seven days of culture. It is possible that the regeneration occurred because the lamina supplied endogenous hormones and nutrients to the petiole which possessed good regeneration capacity.

Callus was initiated at the cut end of the petiole with shoots and roots forming subsequently. In addition, some explants produced embryogenic callus, which, in some cases produced cotyledonal stage embryos within 3 weeks. NAA induced initiation of embryogenesis as reported previously (Lazzeri et al., 1985; Ghazi et al., 1986). However, this is the first report of somatic embryogenesis obtained from cotyledonal explants of *Brassica* species.

Various NAA and BAP concentrations influenced organogenesis of *B. napus* from cotyledonal explants. Medium 3 (10 mg/l NAA) gave the highest
shoot regeneration followed by medium 6 (0.25 mg/l BAP) (Table 4.2). The
difference between the two treatments was that NAA supported direct
organogenesis, shoot proliferation via callus formation and somatic
embryogenesis, but BAP induced only direct organogenesis. However, NAA
(low) and BAP (high) concentrations within the same medium (media 7, 8, 9 and
10) did not improve shoot regeneration (Table 4.4).

In previous work, high BAP and low NAA concentrations have given the
highest level of shoot regeneration from mature cotyledonary explants of
Brassica species (Narasimhulu and Chopra, 1988; Moloney et al., 1989;
Hachey et al., 1991; Khehra and Mathias, 1992). MS medium without growth
regulators (medium 1) also gave good shoot regeneration (67%, 4.60 shoots
per explant). This showed that immature cotyledons already have sufficient
endogenous hormone to initiate organogenesis without adding any growth
regulator in vitro. In contrast, the induction of regeneration from mature
cotyledonary explants from Brassica species required a combination of auxin
and cytokinin growth regulators (Murata and Orton, 1987; Narasimhulu and

Somatic embryogenesis from immature cotyledon explants was
investigated using 5 mg/l NAA medium and high frequency of embryogenesis
was obtained. Two types of somatic embryogenesis were obtained from the
same experiment - direct embryogenesis and embryogenesis with intermediate
callus stage. It was reported that in vitro somatic embryos could initiate either
from callus or directly from tissue without intermediate callus stage in vitro
(Sharp et al., 1980; Williams and Maheswaran, 1986). Direct somatic
embryogenesis has been reported for various plant species, Arachis hypogea
(Hazra et al., 1989), Brassica nigra (Narasimhulu et al., 1992) and Capsicum
annuum (Harini and Lakshmi Sita, 1993). In the present work, embryos initiated
directly from the epidermis of cotyledonary margins. This observation was in
agreement with previous reports (Dos Santos et al., 1983; Gill et al., 1993).
Embryos were tubular structure as reported by Narasimhulu et al. (1992).
Second type of embryogenesis occurred via a callus formation which was initiated at the cut end of the cotyledonary petiole. The appearance of early cotyledonary embryos was later than the appearance of direct embryos due to the intervening callus stage. Maheswaran and Williams (1984) reported that plants regenerated from direct somatic embryogenesis were more uniform than plants produced via callus.

In conclusion, immature cotyledons of *B. napus* gave high efficiency of adventitious shoot regeneration and somatic embryogenesis. Direct organogenesis could be very useful for *in vitro* micropropagation of *B. napus*. Somaclonal variation from regenerated plants could be less than in mature cotyledon explants because of the rapid and direct organogenesis (Larkin and Scowcroft, 1981). Somatic embryogenesis and organogenesis via callus phase may be useful for *Agrobacterium*-mediated transformation of *B. napus*, which is highly inefficient at present.

4.4 Summary

Rapid and high frequency shoot regeneration was achieved from cotyledonal explants of *B. napus*. Two types of cotyledonary explants - 2/3 distal cotyledons and cotyledon and petiole, were cultured on media containing various plant growth regulators. MS medium supplemented with 2 mg/l BAP + 0.5 mg/l NAA gave the highest adventitious shoot regeneration (80%). Media without NAA, but containing concentrations BAP at 2, 4 or 6 mg/l failed to initiate shoot regeneration. Shoot regeneration initiated via callus formation on the cut surface of the cotyledons. In cotyledon+petiole explants, the highest adventitious shoot regeneration (90%) was obtained from 4 mg/l BAP + 2 mg/l kinetin + 0.10 mg/l NAA. Organogenesis occurred at the cut end of the cotyledonary petioles. Cotyledon+petiole explants were used for *Agrobacterium*-mediated transformation. Of the wild-type oncogenic strains only the C58 nopaline strain gave tumours effectively. Transformed shoots were obtained from *B. napus* cv Cobra using disarmed C58C1 (pGV3850) :: pBI121.
via kanamycin selection.

Immature cotyledon of *Brassica napus* cv. Topaz were used to investigate the effects of various NAA (auxin) and BAP (cytokinin) concentrations on morphogenesis *in vitro*. A high efficiency of shoot regeneration and somatic embryogenesis was achieved in Murashige and Skoog medium (1962) supplemented with growth regulators. 10 mg/l NAA and 0.25 mg/l 6-benzylaminopurine gave the best shoot proliferation at 7.3 and 6.8 shoots per explant, respectively. Such explants also produced a number of shoot promordia. Adventitious shoots were initiated either directly from subepidermal tissues of cotyledonary petioles (direct organogenesis) or via callus formation at the cut end of the petiole. Somatic embryogenesis was obtained with 5 mg/l, 10 mg/l and 40 mg/l NAA acid with transfer to medium without growth regulators.
CHAPTER 5
ANTHER-SPECIFIC A1 CDNA FROM B. NAPUS SHOWS SIMILARITY TO CHALCONE AND STILBENE SYNTHASE SEQUENCES

5.1 Introduction
The A1 cDNA was isolated from the B. napus cDNA library constructed from anthers of 1.2-1.8 mm buds. A1 is a high abundance clone isolated by differential screening (Scott et al., 1991a). A1 mRNA is expressed in young anthers during the tetrad and microspore release stages of microsporogenesis. When the A1 cDNA was sequenced the predicted peptide sequence was found to show similarity to chalcone synthase (CHS) and stilbene synthase (STS) enzymes.

CHS and STS represent a closely related group of enzymes in both structure and function. Both enzyme types are able to use the same substrates, but generate different products (Schröder et al., 1988). Many chalcone synthase genes and cDNAs have been cloned and sequence information is available.
from a wide range of species including the gymnosperm *Pinus sylvestris* (Fliegmann *et al*., 1992), monocots such as maize (Franken *et al*., 1991) and barley (Rohde *et al*., 1991) and several dicot species including the well characterised petunia family (Koes *et al*., 1990), *Arabidopsis* (Feinbaum and Ausubel, 1988) and tomato (O’Neill *et al*., 1990). In contrast, only 3 STS sequences have been described: resveratrol synthase from *Arachis hypogaea* (Schröder *et al*., 1988); dihydropinosylvin synthase from *P. sylvestris* (Fliegmann *et al*., 1992); and stilbene synthase from grapevine (Melchior and Kindl, 1990).

5.2 Results

5.2.1 The A1 cDNA is Incomplete

Total RNA was isolated from 1-2 mm long buds of *B. napus* and hybridized with the A1 cDNA probe after gel blotting. Northern gel blot hybridization showed that the probe hybridized to transcripts of approximately 1.6 kb in size (Figure 5.1). Since the A1 cDNA clone used in the present work was shown to be approximately 700 bp long, this result indicated that the cDNA was truncated. This was confirmed by nucleotide sequencing which showed that the cDNA was 725 bp long and that the predicted open reading frame extended to up to the extreme 5' end of the cDNA (Figure 5.2). Since antisense RNA expression is most likely to succeed with a full length RNA attempts were made to obtain a full length cDNA by further screening of the cDNA library using the A1 cDNA as a probe. Rescreening identified several positive plaques. However, despite several attempts, none proved to be an improvement over the original clone in terms of length. An *Arabidopsis* genomic library was also screened, but again this proved unsuccessful.
**Figure 5.1**

Northern blots of bud RNA probed with A1 cDNA to show the temporal expression patterns of the 2 transcripts. 10μg of total RNA were loaded per lane. Lane 1, <1 mm buds; lane 2, 1-2 mm buds; lane 3, 2-3 mm buds; lane 4, 3-4 mm buds; lane 5, 4-5 mm buds; lane 6, >5 mm buds (lane 5 had contamination). Autoradiographs were exposed for 2 days.
Figure 5.2
DNA sequence and predicted amino-acid sequence of the A1 cDNA. A putative polyadenylation signal is underlined, at position 602-607. Sequencing was carried out using a T7 DNA polymerase sequencing kit which employs dideoxynucleotide chain-termination mixes, as described by Sanger et al. (1977).
5.2.2 A1 Shows Similarity to Chalcone and Stilbene Synthases

Although the A1 cDNA does not represent full-length mRNA its amino acid sequence shows similarity to published CHS and STS sequences (Figure 5.3). The A1 peptide also shows significant similarity (81% identity at the amino acid level) to the protein encoded by the BA42 transcript, an anther-specific mRNA isolated from B. napus (Shen and Hsu, 1992). These authors suggested that BA42 encodes a diverged CHS, but do not comment on the similarity to stilbene synthase. The degree of similarity between BA42 and A1 suggests that they may be the result of a relatively recent gene duplication event. *In situ* hybridization experiments showed that the BA42 message is present in the tapetum and vascular tissue of immature anthers and also in young microspores (Shen and Hsu, 1992). Figure 5.3 is an alignment of the A1 peptide with BA42, and CHS and STS sequences from gymnosperms, monocots and dicots. Figure 5.4 is a dendrogram based on the alignment in Figure 5.3. It is probable that A1/BA42, CHS and STS sequences share a common ancestor, but the anther-specific sequences are a distinct group which diverged much earlier than the CHS/STS family.

5.2.3 Construction of A1 Sense and Antisense Chimaeric Genes

The A1 cDNA was isolated from *B. napus* anther cDNA library which was constructed in lambda ZapII (Stratagene) by the ligation of EcoRI, NotI linkers onto the ends of the cDNA (Scott *et al.*, 1991a). The *B. napus* A1 cDNA was cloned in both orientations into the NotI site of pWP80, forming pWP80A1A (antisense) and pWP80A1B (sense). Construction of pWP80 is explained in Chapter 6. pWP80 contains a 936 bp *A. thaliana* A9 promoter fragment and a polylinker region followed by a 785 bp CaMV polyadenylation sequence. The WP80A1 chimaeric genes were then cloned as HindIII, XhoI fragments into HindIII, SalI - cut pBin19 (Bevan, 1984) forming pWP80A1ABin and pWP80A1BBin (Figure 5.5) and mobilised into *A. tumefaciens* strain pGV2260 via triparental mating. pGV2260/pWP80A1Bin strains were recovered in...
Figure 5.3
Alignment of the predicted amino-acid sequences of A1 with CHS and STS sequences and the anther-specific sequence BA42. Sequences were aligned using the program CLUSTAL. Asterisks identify residues shared by all sequences, dots indicate conservative substitutions. BA42 Bn, *B. napus* anther-specific mRNA (Shen and Hsu, 1992); C19 Bn, *B. napus* (Hodge et al., 1992); CHS Sa, mustard chalcone synthase (Batschauer et al., 1991); CHS Sc, parsley chalcone synthase (Reimold et al., 1983); CHS Gm, soybean chalcone synthase gene 3 (Akada et al., 1990); CHS Ps, *P. sylvestris* chalcone synthase (Fliegmann et al., 1992); CHS Zm, maize chalcone synthase Whp gene (Franken et al., 1991); STS VO, grapevine stilbene synthase (Melchior and Kindl, 1990); STS Ah, peanut resveratrol synthase (Schröder et al., 1988); STS Ps, *P. sylvestris* dihydropinosylvin synthase (Fliegmann et al., 1992).
Figure 5.4
Dendrogram based on the alignment in Figure 5.3. BA42 Bn, *B. napus* anther-specific mRNA (Shen and Hsu, 1992); C19 Bn, *B. napus* (Hodge *et al.*, 1992); CHS Sa, mustard chalcone synthase (Batschauer *et al.*, 1991); CHS Sc, parsley chalcone synthase (Reimold *et al.*, 1983); CHS Gm, soybean chalcone synthase gene 3 (Akada *et al.*, 1990); CHS Ps, *P. sylvestris* chalcone synthase (Fliegmann *et al.*, 1992); CHS Zm, maize chalcone synthase Whp gene (Franken *et al.*, 1991); STS VO, grapevine stilbene synthase (Melchlor and Kindl, 1990); STS Ah, peanut resveratrol synthase (Schröder *et al.*, 1988); STS Ps, *P. sylvestris* dihydropinosylvin synthase (Fliegmann *et al.*, 1992).
% similarity between amino-acid sequences

A1
BA42
CHS Sa
CHS Pc
CHS Gm
CHS Ps
CHS Zm
STS VO
STS Ah
STS Ps
selective medium containing 50 μg/ml kanamycin, 50 μg/ml rifampicin and 10 μg/ml ampicillin.

The constructs, pWP80A1ABin and pWP80A1BBin, were used to transform tobacco to determine if these constructs express *B. napus* A1 RNA. Both constructs expressed *B. napus* A1 RNA, though sense RNA levels were much higher than antisense levels (Figure 5.6). However, these sense and antisense constructs were not used to transform *A. thaliana* and *B. napus* because the A9 promoter is active only in the tapetal cells and it is thought that A1 expresses not only in the tapetum, but also in vascular tissue and young microspores. The CaMV 35S promoter is active only in the vascular bundle of the anther (see Chapter 4), therefore it was not suitable to drive A1 antisense RNA expression.

5.3 Discussion
The stilbene synthases (STS) are key enzymes in the synthesis of stilbene-type phytoalexins. Resveratrol synthase catalyses the condensation of 4-coumaroyl with 3 malonyl-CoA molecules to form resveratrol and other STS enzymes similarly catalyse the formation of pinoresinol and dihydropinoresinol. Stilbenes are present in a limited number of plant species and their presence is usually associated with stress or fungal attack.

Chalcone synthase (CHS) is the key enzyme in the flavonoid biosynthesis pathway. The enzyme catalyses a condensation of a 4-coumaroyl-CoA with 3 malonyl-CoA molecules to form naringenin chalcone or similarly uses cinnamoyl-CoA to form pinocembrin chalcone. Flavanoids are known to be involved in many plant processes including pigmentation, defence, and U.V. protection and the observed expression of the genes described reflects these roles. In some of the species studied ie *Petunia hybrida*, chalcone synthase is a multi-gene family and it has been shown in *P. hybrida* (Koes et al., 1990) and *Zea mays* (Franken et al., 1991) that that individual CHS genes show different expression patterns suggesting that, at least in some species, the different
Figure 5.5
Schematic representation of A1 sense and antisense chimaeric genes used in tobacco transformation. The *B. napus* A1 cDNA (725 bp) was cloned in both orientations into the *Ncol* site of pWP80 which contains a 936 bp *A. thaliana* A9 promoter and a polylinker region followed by a 785 bp CaMV polyadenylation sequence, forming pWP80A1A (antisense) and pWP80A1B (sense). The WP80A1 chimaeric genes were then cloned as *HindIII, XhoI* fragments into *HindIII, SalI*-cut pBin19 (Bevan, 1984) forming pWP80A1ABin and pWP80A1BBin.
HindIII Xho/Sall

A9 promoter A1 cDNA CaMV poly A pWP80A9ABin

200 bp

Promoter region CaMV polyadenylation sequence A1 cDNA
Figure 5.6
Expression of the A1 sense and antisense chimaeric genes in transgenic tobacco anthers dissected from 10-12 mm long buds. Lanes 1-3, transformed tobacco plants with pWP80A1BBin (sense); Lanes 4-6, transformed tobacco plants with pWP80A1ABin (antisense).
members of the gene family may have specific roles.

In two of the species studied, CHS has been shown to be expressed in male reproductive tissue. In *P. hybrida*, the *chsA*, *chsJ* and *chsB* genes were shown, via promoter-GUS fusions, to be expressed in the vascular tissue and the tapetum of young anthers (Koes *et al.*, 1990). The *Whp* gene of *Zea mays* is expressed in the tassels (male flowers) (Franken *et al.*, 1991). Recently, Van der Meer *et al.*, (1992) demonstrated that flavanoids are essential for pollen development. A modified CaMV 35S promoter was used to drive an antisense CHS gene in transgenic petunia plants. This construct inhibited pigment synthesis in the anther and transgenic plants with white anthers were male sterile due to an arrest in male gametophyte development. Similarly, maize plants that are double mutants at the CHS loci *C2* and *Whp* have white pollen and are male sterile (Franken *et al.*, 1991).

Another cDNA clone C19 was also isolated from the same *B. napus* cDNA library constructed from anthers of 1.2-1.8 mm buds. Unlike the A1 cDNA, C19 represents a low abundance transcript identified via cold plaque screening (Hodge *et al.*, 1992). Both cDNA clones, A1 and C19, show 44% identity to one another at the amino acid level. The temporal and spatial expression patterns of the A1, C19 and BA42 transcripts suggests they may represent highly diverged CHS sequences. There are examples of anther-specific genes which are significantly diverged from their vegetative equivalents such as B-tubulins (Hussey *et al.*, 1988) and *Adh-1* (Schwartz, 1971). However in petunia, apparently all CHS expression in the anther is encoded by genes which are also expressed in the corolla, flower tube, flower stem, ovaries, seedpods and on U.V. induction. In contrast, the expression of A1, BA42 and C19 transcripts is restricted to the anther suggesting that they do not represent functional equivalents of the petunia genes. This discrepancy could be explained if there are additional anther-specific genes in petunia which represent equivalents of A1 / C19 / BA42 family, but do not cross-hybridize with CHS. If equivalents of A1, C19 and BA42 exist in petunia, it is likely, considering the divergence in
nucleotide sequence between these transcripts and CHS, that the CHS anti-
sense transformants would retain wild-type A1, C19 and BA42 transcript levels. 
However, CHS antisense plants were male-sterile which suggests that petunia 
equivalents of A1, C19 and BA42 gene expression can not substitute for an 
absence or reduction in CHS transcript levels, and hence that A1, C19 and 
BA42 do not encode CHS proteins. Alternatively, anther-specific CHS genes 
may only exist in some species, although this seems unlikely.

There is no data on the expression of STS genes or the presence of 
stilbenes in the anther and it is therefore impossible to speculate whether A1, 
C19 and BA42 could represent STS sequences. However, it has been noted 
that STS sequences appear to show less similarity to one another than CHS 
sequences (Schöder et al., 1988), and this is clearly the case for the 3 STS 
sequences available, as shown in Figure 5.4.

5.4 Summary

The A1 cDNA clone represent anther-specific transcripts whose predicted 
peptide sequence show similarity to those of the enzymes chalcone synthase 
(CHS) and stilbene synthase (STS). A1 also shows strong similarity to another 
B. napus anther-specific transcript BA42 (Shen and Hsu, 1992). Together with 
BA42, A1 and C19 (Hodge et al., 1992) represent a family of B. napus anther-
specific genes which encode proteins related to the CHS/STS group but which 
are considerably different from them. It is not known whether the proteins 
encoded by these genes carry out CHS or STS type reactions in vivo, or 
whether they catalyse one or more other related but distinct type(s) of reaction.
CHAPTER 6

THE HIGHLY EXPRESSED TAPETUM-SPECIFIC A9 GENE IS NOT REQUIRED FOR MALE FERTILITY IN BRASSICA NAPUS

6.1 Introduction

The microsporocytes and tapetal cells are the most important cell-types within the anther. The significance of the tapetal cells in anther development is demonstrated by the fact that natural male sterility is often linked to tapetal malfunction (Kaul, 1988). Recently many workers have reported the isolation of cDNAs representing anther-specific or anther-prevalent transcripts expressed during microsporogenesis (Twell et al., 1989; Hanson et al., 1989; Scott et al., 1991a; Albani et al., 1991; Theerakulpisut et al., 1991; Shen and Hsu, 1992; Roberts et al., 1993a; Roberts et al., 1993b). Although some of these transcripts
potentially encode polypeptides which exhibit homology to previously characterised sequences, the functions of most of these anther proteins remains unclear. One example is the tapetum-specific B.napus A9 protein which displays homology to a family of seed proteins (Paul et al., 1992). This family contains proteins that are seed storage proteins and protease inhibitors. The A9 cDNA clone, isolated from a B. napus anther cDNA library, represents a highly abundant tapetum-specific transcript (estimated at 0.2% of anther message) (Scott et al., 1991a). The transcript is confined to the tapetum of meiocyte to microspore interphase stage anthers.

One approach to addressing the roles of these anther-specific genes is to study the phenotypes of plants in which the expression of these genes has been downregulated or abolished. A useful technique to achieve this objective is the expression of antisense RNA in transgenic plants. When expressed in transgenic plants antisense RNA with a complementary sequence to a specific mRNA has been shown to effectively downregulate the expression of the target plant gene (Smith et al., 1988; Van der Krol et al., 1988; Van der Meer et al., 1992) and has led to the identification of proteins encoded by the downregulated transcript (Hamilton et al., 1990; Bird et al., 1991). In this work i attempted to determine the role of the A9 gene of B.napus (Scott et al., 1991a) by the expression of A9 antisense RNA in transgenic plants. Since localization of expression of A9 transcript in tapetal cells has been determined by in situ hybridization (Scott et al., 1991a). The B. napus A9 cDNA and the A. thaliana A9 gene encoded proteins which were 73% identical and were predicted to be 10.3 kDa and 11.6 kDa in size respectively (Paul et al. 1992). Alignment of the putative polypeptides encoded by the B. napus A9 cDNA and A. thaliana A9 gene are shown in Chapter 7. A9 promoter isolated from Arabidopsis genomic library was fused to the barnase (Bacillus amyloliquefaciens RNase) and the reporter gene β-glucuronidase showed that A9 promoter is active in tapetal cells (Paul et al., 1992). Although CaMV 35S promoter has been used to drive antisense expression in most cases (Table 6.1), the A. thaliana A9
Table 6.1 Promoters used to drive antisense genes for downregulation of target genes

<table>
<thead>
<tr>
<th>Target</th>
<th>Promoter</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A9</td>
<td>A9</td>
<td><em>B. napus</em></td>
<td>Present work</td>
</tr>
<tr>
<td>CHS (corolla)</td>
<td>CaMV 35S</td>
<td><em>Petunia hybrida</em>, <em>N. tabacum</em></td>
<td>Van der Krol <em>et al.</em> 1988</td>
</tr>
<tr>
<td>Polygalacturonase</td>
<td>CaMV 35S</td>
<td><em>Lycopersicon esculentum</em></td>
<td>Smith <em>et al.</em>, 1988</td>
</tr>
<tr>
<td>RUBISCO</td>
<td>CaMV 35S</td>
<td><em>N. tabacum</em></td>
<td>Rodermel <em>et al.</em> 1988</td>
</tr>
<tr>
<td>CHS (corolla)</td>
<td>CHS, CaMV 35S</td>
<td><em>P. hybrida</em></td>
<td>Van der Krol <em>et al.</em> 1990</td>
</tr>
<tr>
<td>pTOM13</td>
<td>CaMV 35S</td>
<td><em>L. esculentum</em></td>
<td>Hamilton <em>et al.</em> 1990</td>
</tr>
<tr>
<td>pTOM5</td>
<td>CaMV 35S</td>
<td><em>L. esculentum</em></td>
<td>Bird <em>et al.</em> 1991</td>
</tr>
<tr>
<td>CHS (anther)</td>
<td>CaMV 35S + Anther Box</td>
<td><em>P. hybrida</em></td>
<td>Van der Meer <em>et al.</em> 1992</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>CaMV 35S</td>
<td><em>Daucus carota</em></td>
<td>Gogarten <em>et al.</em> 1992</td>
</tr>
<tr>
<td>Stearoyl-ACP</td>
<td>CaMV 35S</td>
<td><em>B. napus</em>, <em>B. rapa</em></td>
<td>Knutson <em>et al.</em> 1992</td>
</tr>
<tr>
<td>Desaturase</td>
<td>CaMV 35S</td>
<td><em>N. sylvestris</em></td>
<td>Neuhaus <em>et al.</em> 1992</td>
</tr>
<tr>
<td>6-1,3-glucanase (leaf)</td>
<td>CaMV 35S</td>
<td><em>C. nicotianae</em></td>
<td>Neuhaus <em>et al.</em> 1992</td>
</tr>
<tr>
<td>Pectin esterase</td>
<td>CaMV 35S</td>
<td><em>L. esculentum</em></td>
<td>Hall <em>et al.</em> 1993</td>
</tr>
</tbody>
</table>
promoter was chosen in present work. This was because the CaMV 35S promoter has not proven to be active in tapetum cells (see Chapter 4) and A9 promoter was available which would ensure a perfect match between antisense and target RNA. The \textit{B.napus} A9 cDNA was linked in both sense and antisense orientations to the \textit{A. thaliana} A9 promoter (Paul \textit{et al.}, 1992) and the chimaeric genes transferred to tobacco, \textit{A. thaliana} and \textit{B.napus}.

6.2 Results

6.2.1 Construction of A9 Sense and Antisense Chimaeric Genes

The \textit{B.napus} anther cDNA library from which A9 was isolated was constructed in lambda ZapII (Stratagene) by the ligation of EcoRI, NotI linkers onto the ends of the cDNA (Scott \textit{et al.}, 1991a). Consequently, the \textit{B.napus} A9 cDNA could be isolated as a 490 bp NotI fragment which was then cloned in both orientations into the \textit{NotI} site of pWP80 and pWP83, forming pWP80A9A (antisense) and pWP80A9B (sense), pWP83A9A (antisense) and pWP83A9B (sense). pWP80 was constructed from pWP72 which carries an A9 \textit{A. thaliana} promoter fragment (Paul \textit{et al.}, 1992). pWP72 was cut with \textit{XbaI} and religated to remove a \textit{BamHI} site forming pWP78. The \textit{KpnI}, \textit{SstI} (blunted) fragment of pWP78 was then cloned into \textit{KpnI}, \textit{SmaI}-cut pJiT60 (Guerineau \textit{et al.}, 1988) forming pWP80. pWP80 contains a 936 bp \textit{A. thaliana} A9 promoter fragment and a polylinker region followed by a CaMV polyadenylation sequence. pWP83, which carries a 785 bp CaMV 35Sx2 promoter fragment and CaMV polyadenylation sequence, was constructed using pWP80. \textit{KpnI}, \textit{XbaI} CaMV 35S promoter fragment of pJIT30 was cloned into \textit{KpnI}, \textit{XbaI} - cut pWP80 which removes A9 promoter fragment. \textit{KpnI}, \textit{HindIII} fragment of pJIT80 which has double 35S promoter was cloned into \textit{KpnI}, \textit{HindIII} - cut pWP82 to create pWP83. The WP80A9 chimaeric genes were then cloned as \textit{HindIII}, \textit{XhoI} fragments into \textit{HindIII}, \textit{SalI} - cut pBin19 (Bevan, 1984) forming pWP80A9ABin and pWP80A9BBin, and the WP83A9 chimaeric genes were cloned as \textit{KpnI}, \textit{XhoI} fragments into \textit{KpnI}, \textit{SalI} - cut
Figure 6.1
Schematic representation of A9 sense and antisense chimaeric genes used in tobacco, Arabidopsis and B. napus transformation. Small arrows represent the location of the PCR primers. A 490 bp NotI fragment of A9 cDNA was cloned in both orientations into the NofI site of pWP80 which carries a 936 bp A. thaliana promoter fragment and a polylinker region followed by a CaMV polyadenylation sequence and pWP83 which carries a 785 bp CaMV 35Sx2 promoter fragment.

A. The WP80A9 chimaeric genes were cloned as HindIII, SalI fragments into HindIII, SalI-cut pBin19 (Bevan, 1984).

B. The WP83A9 chimaeric genes were cloned as KpnI, XhoI fragments into KpnI, SalI-cut pBin19.
A

HindIII → A9 promoter → 126' → RSPL

126' → Xhol/Sall → M13'20

pWP80A9ABIn

RSPL → A9 promoter → 240' → CaMV poly A

200 bp

B

Kpnl → p35Sx2 → 126' → RSPL

126' → Xhol/Sall → M13'20

pWP83A9ABIn

RSPL → p35Sx2 → 240' → CaMV poly A

200 bp

Promoter region

A9 3' noncoding region

A9 coding region

CaMV polyadenylation sequence
pBin19 (Bevan, 1984) forming pWP83A9ABin and pWP83A9BBin (Figure 6.1). These binary plasmids were then mobilised into A. tumefaciens strain pGV2260 via triparental mating (Draper et al., 1988). pGV2260 / pWP80A9Bin and pWP83A9Bin strains were recovered on selective medium containing 50 μg/ml kanamycin, 50 μg/ml rifampicin and 10 μg/ml ampicillin.

6.2.2 Expression of A9 Sense and Antisense Chimaeric Genes In Tobacco

A 490 bp B.napus A9 cDNA fragment was linked in both orientations to the A.thaliana A9 promoter and CaMV 35Sx2 promoter as shown in Figure 6.1. These constructs were then used to transform tobacco. The A.thaliana A9 promoter, isolated using the B.napus A9 cDNA (Paul et al., 1992), has an identical spatial and temporal pattern of expression to the B.napus A9 transcript. Thus, in the absence of a cloned B.napus A9 promoter, the A.thaliana promoter is ideal for the expression of A9 RNA in B.napus. The double CaMV 35S promoter which is widely used for plant transformation, was used as a constitutive promoter for comparative purposes. Since the B.napus A9 cDNA does not hybridize to tobacco anther RNA (Personal communication, R. Hodge, Leicester), the A9 antisense and sense chimaeric genes were initially transformed into tobacco to determine if these constructs express B. napus A9 RNA. Both constructs express B. napus A9 RNA, though sense RNA levels were considerably higher than antisense levels. Such an effect has been observed previously, leading to the suggestion that translatable RNA is more stable in plants than inefficiently translated RNA (Vancanneyt et al., 1990). It may be significant that the sense construct is predicted to be translated, producing the putative A9 protein. The A9 sense plants expressed two A9 RNA transcripts; the smaller transcript, which is absent in the antisense plants, probably results from transcriptional termination at an A9 polyadenylation sequence rather than at the CaMV terminator. All the tobacco plants were phenotypically normal and fully
male fertile which was anticipated given that *B. napus* A9 RNA does not hybridize to tobacco RNA. However, these results confirm that the constructs are capable of expressing both sense and antisense A9 RNA.

### 6.2.3 Analysis of Transgenic *A. thaliana* Plants

*B. napus* A9 cDNA clone and the corresponding *A. thaliana* A9 gene are 76% identical at the nucleotide level and 73% identical at the amino acid level (Paul et al., 1992). This high level of homology between *A. thaliana* A9 gene and A9 cDNA suggested that A9 sense/antisense constructs could be used to downregulate A9 mRNA of *A. thaliana*. Accordingly, the *A. thaliana* ecotype C24 was transformed with A9 sense/antisense constructs, pWP80A9ABin, pWP80A9BBin, pWP83A9ABin and pWP83A9BBin as explained in materials and methods (Chapter 2). Cotyledon explants were co-cultivated for 3-4 days with pGV2260 *Agrobacterium* strains containing the above binary plasmids. Regeneration and selection was carried out in MS medium containing 1 mg/l BAP and 0.1 mg/l NAA, supplemented with 50 mg/l kanamycin and 400 mg/l augmentin. 15 pWP80A9ABin (antisense), 10 pWP80A9BBin (sense), 18 pWP83A9ABin (antisense) and 17 pWP83A9BBin (sense) plants were recovered on kanamycin selection medium and pollen development was investigated by light microscopy. Number and shape of pollen grains from transformed plants looked normal as wild-type *Arabidopsis* plants. All transformants were apparently vegetatively normal and set normal levels of seed, as judged by silique elongation, when allowed to self. T2 plants from initial transformants were obtained by selfing and germinating seeds on kanamycin medium. Pollen development of the T2 plants also showed that A9 sense and antisense RNAs had no obvious effect on male fertility of *A. thaliana*. 

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6.2.4 Agrobacterium-Mediated Transformation and Analysis of Transgenic *B. napus* Plants

Cotyledons were removed from 5-day old *B. napus* cv Westar seedlings and co-cultivated with pGV2260 *Agrobacterium* strains containing binary plasmids (pWP80A9ABin and pWP80A9BBin) on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 4 mg/l 6-benzylaminopurine (BAP) as explained in material and methods. After 3 days, the cotyledons were transferred to regeneration medium containing 4 mg/l BAP, 15 mg/l kanamycin and 400 mg/l augmentin. Regenerated shoots were grown on MS medium without growth regulators then rooted shoots were transferred to soil.

Four *B. napus* transformants (3B, 3D, 4A and 4B) containing the antisense construct were recovered from two different explants, and a single A9 sense plant was produced. Southern blot hybridization was performed to confirm that these T1 plants were transformed (data not shown). PCR was used to follow the inheritance of the transferred DNA through the T2 and T3 generations. Progeny of the T1 plants (T2 and T3 generations) were checked by PCR using 5'-126 and M13'20 primers and 3'-240 and RSPL (long reverse primer) primers (Figure 6.2). Figure 6.2 shows that DNA from T3 A9 antisense plants gave the predicted fragment of about 1.3 kb when using 3'-240 and RSPL (long reverse primer) primers. Similarly, the RSPL and M13'20 primers were used to confirm that the sense plant was transformed.

Original transformants were observed for seed production. 3B and 3D exhibited very low numbers of seed per silique while 4A and 4B showed reduced number of seed per silique. Scanning electron microscopy of 3B and wild-type *B. napus* also demonstrated that the pollen of 3B was morphologically abnormal (Figure 6.6). Thus, the progeny of the primary transformants, resulting from selfing or crossing to wild-type (cv Topaz), were analysed to determine if reduced fertility segregated with the A9 antisense gene and was correlated with a reduction in A9 transcript levels. PCR analysis of 65 T2 plants (5 selfed + 21 crossed with wild-type from 3B, 3 selfed + 20 crossed from 4B, 3 selfed + 8
Figure 6.2

PCR analysis of T3 antisense transformed *B. napus* plants using 3'-240 and RSPL (long reverse primer) primers. Lane 1, DNA size marker; Lane 2, pWP80A9A plasmid; Lane 3, wild-type *B. napus*; Lanes 4-20, A9 antisense *B. napus* plants. Lanes 8 and 16 (4B/56x-2 and 3D/49s-5) appear to be PCR negative as well as wild-type *B. napus*. Arrow indicates expected size of PCR product as 1.3 kb.
crossed from 3D and 2 selfed + 3 crossed from 4A) showed that all contained the A9 antisense gene. In a total of 75 T3 plants, produced by selfing or outcrossing to wild-type, only 10 plants lacked the antisense gene (PCR-negative) (indicated in Table 6.2). Thus it appears that the A9 antisense gene was present in more than one loci in each of the primary transformants. Since the T2 progeny contained no plants that were PCR negative detailed analysis was performed on the T3 progeny.

6.2.5 A9 Transcript Levels are Severely Reduced in A9 Antisense Plants

RNA was extracted from 1.5 - 2.5 mm buds of wild-type, PCR positive and PCR negative A9 sense and antisense T3 plants. During this developmental period the A9 transcript is most abundant (Scott et al., 1991a). Duplicate RNA blots were either probed with $^{32}$P-labeled B.napus A9 cDNA or with a B.napus A3 cDNA probe. The A3 cDNA was isolated from the same B.napus library as the A9 cDNA and represents a tapetum-specific transcript with a temporal expression pattern indistinguishable from that of A9 (Scott et al., 1991a; Scott et al., 1991b). Although an abundant transcript, A3 transcript levels are less than those of A9. Therefore, the ratio of the signals obtained by probing with A3 and A9 should indicate if the A9 transcript is down-regulated in the transgenic plants. Representative duplicate gel blots are shown in Figure 6.3. The wild-type and transgenic plants all accumulated A3 transcript, but in a total of 8 A9 antisense plants only one (4B/72s-4) accumulated significant levels of A9 mRNA; the remainder had severely reduced or, in the case of plant 3D/5s-1, undetectable A9 transcript levels. As expected, the A9 sense plant expressed more A9 transcript in comparison to A3 levels than the wild-type plant (Figure 6.3). PCR negative plants (3D/49s-5) showed normal levels of A9 transcript (Figure 6.3). Thus the presence of the A9 antisense gene was almost always associated with a decrease in A9 transcript levels. Densitometry on the original autorads demonstrated that A9 mRNA levels in some transformants (3D/5s-1, 4B/53s-1
Figure 6.3
Northern gel blot analysis of antisense *B. napus* plants. (a) Hybridized with an A3 cDNA probe which is also tapetum-specific and shows an identical temporal expression pattern to that of A9 cDNA, (b) Hybridized with A9 cDNA probe. Lane 1, wild-type *B. napus*; Lane 2, 4B/72s-4; Lane 3, 4B/57x-4; Lane 4, 4B/53s-4; Lane 5, 3D/49s-2; Lane 6, 3D/104x-1; Lane 7, 4B/53s-3; Lane 8, 3D/5s-1; Lane 9, 3D/49s-5, a PCR (-) plant; Lane 10, 3D/104x-5, (c) A9 and A3 transcript levels in wild-type and A9-sense transformed plants. Lanes 1 and 3, wild-type *B. napus*; Lanes 2 and 4, A9-sense transformed plant. Lanes 1 and 2, hybridized with A9 cDNA probe; Lanes 2 and 3, hybridized with A3 cDNA probe.
Figure 6.4

Northern dot blot analysis of wild-type and two antisense *B. napus* plants (3D/5s-1 and 3D/104x-1) which showed reduced level of A9 mRNA with Northern gel blot. Total RNA from a range of bud lengths were hybridized with A9 cDNA probe. A. 5 µg of total RNA sample was spotted onto per square of Hybond-N and then hybridized with a ribosomal probe, B. A9 cDNA probe.
Bud length (mm)
Figure 6.5
Temporal pattern of A9 transcript from antisense transformed (3D/5s-1 and 3D/104x-1) and wild-type B napus plants. Data were obtained using densitometry of dot blots.
and 4B/57x-4) were about 5% of that in the wild-type and PCR negative (3D/49s-5) plants. However, 4B/72s-4 showed 65% A9 mRNA level of that in the wild-type plant. It is possible that the few A9-antisense plants that accumulated significant levels of A9 either contain fewer A9 antisense gene copies or possess copies that are weakly expressed.

To confirm that A9 expression is downregulated in A9 antisense plants throughout the developmental sequence of microsporogenesis, RNA from a range of bud lengths was isolated from wild-type and antisense plants. RNA dot blots were hybridized with labelled A9 cDNA probe and a ribosomal probe to confirm equal loading of RNA (Figure 6.4). The A9 transcript was present in wild-type buds of length 1-3 mm. However, two antisense plants (3D/5s-1 and 3D/104x-1) which showed severely reduced levels of A9 transcript on RNA gel blots, did not express detectable levels of A9 message at any developmental stage (Figure 6.4, Figure 6.5).

6.2.6 Reduced Pollen Viability is not Correlated with Downregulation of A9 Transcript Levels

All the transgenic T3 B.napus plants produced siliques which carried 10-30 seeds per silique after self- and cross-pollination (Table 6.3). However, analysis of pollen viability (see Materials and Methods) revealed variation in the male fertility of plants. When stained with the mixture developed by Alexander (1969), viable pollen grains stained red and aborted pollen grains stained green (Figure 6.7). Almost 100% of wild-type pollen appeared red by staining and was therefore assumed to be viable (Figure 6.7). However, in transformed plants, pollen viability ranged from 60% to 100% (Table 6.2). The aborted green-stained pollen grains were ovoid in shape. The reduction in pollen viability was not correlated with a decrease in A9 message levels since some PCR negative plants exhibited reduced male fertility (80% viable in plant 3D/49s-5; Table 6.2). Some PCR positive plants with severely reduced A9 transcript levels also had normal levels of pollen viability (100% viable in plant 3D/104x-5) (Figure 6.3,
Figure 6.6
Scanning electron microscopy of pollen from wild-type and two A9-antisense plants of *B. napus*. (a) Wild-type, *B. napus* cv Topaz, (b) initial transformant, 3D which exhibited very low numbers of seed per siliqua. However, the reduced male fertility and abnormal pollen development did not transmit to T2 and T3 generations. (c) T3 antisense transformant, 4B/53s-4 which revealed reduced A9 mRNA level due to antisense downregulation and set seed normally.
Figure 6.7
Staining for pollen viability. (a) wild-type *B. napus* plant, (b) T3 antisense transformant, 3D/104x-1. Red and green staining indicates viable and inviable pollen, respectively. x100
Table 6.2 Pollen viability of T3 antisense *B. napus* plants

<table>
<thead>
<tr>
<th>Plant number</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<td>100</td>
</tr>
<tr>
<td>3D/13 x</td>
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<td>92</td>
<td>97</td>
<td>91</td>
<td>80</td>
</tr>
<tr>
<td>3D/29 s</td>
<td>98</td>
<td>98</td>
<td>97</td>
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<tr>
<td>4B/45 s</td>
<td>100</td>
<td>100</td>
<td>89</td>
<td>96</td>
<td>95</td>
</tr>
<tr>
<td>3D/49 s</td>
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<td>80</td>
<td>98</td>
<td>95</td>
<td>80</td>
</tr>
<tr>
<td>4B/53 s</td>
<td>100</td>
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<td>80</td>
<td>59</td>
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<td>97</td>
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* Appeared to be PCR negative (segregant)
1 s=selfed, x=crossed to wild-type
2 *B. napus* cv Topaz
Table 6.2). This data indicates that there is no correlation between levels of pollen viability and the levels of A9 transcript. It is therefore possible that the reduced male fertility observed in some plants may be due to a mutation generated during tissue culture that does not segregate with a T-DNA insertion. However, in most cases despite a severe reduction in A9 mRNA levels in some transformants, the plants exhibit normal male fertility. Also scanning electron microscopy exhibited normal pollen development from T3 transformant, 4B/53s-4 despite primary transformants (3D and 4B) showed abnormal pollen development (Figure 6.6).

The amount of seed set from transformed and untransformed T3 plants was determined by counting the number of seed per silique from selfed and crossed flowers (Table 6.3). Again no correlation was observed between pollen viability and the amount of seed set. For instance 4B/57x-4, which showed 85% pollen viability, produced an average of 9 seeds per silique, but 4B/53s-3 which had a lower pollen viability (70%) produced higher number of seed per silique (17.2) (Table 6.3). In Brassica, the ratio of pollen to ovules is very high (1000s of pollen grains per flower, but an average of 25-30 ovules/silique), therefore a moderate reduction in pollen viability may not cause a marked reduction in seed set.

6.3 Discussion

The discovery that the expression of antisense RNA in cells can result in the downregulation of cognate mRNA has opened up a novel route to determine the function of proteins. Expressing a cDNA in the antisense orientation in transgenic plants has led to the identification of transcripts which encode ACC-oxidase and possibly prephytoene pyrophosphate synthases which is involved in tomato carotenoid biosynthesis (Hamilton et al., 1990; Bird et al., 1991). There are also reports that expression of the sense RNA may also downregulate the corresponding mRNA (Napoli et al., 1990). One of the advantages of the antisense technique is the potential to downregulate transcripts of multigene families, provided that the family of mRNAs is sufficiently homologous. This is of
Table 6.3 Pollen viability and number of seed per silique from transformed T3 plants.

<table>
<thead>
<tr>
<th>Plant number&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Pollen viability (%)</th>
<th>Number of seed/silique</th>
<th>Selfed</th>
<th>Crossed</th>
</tr>
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<td></td>
<td>23.0</td>
<td>25.0</td>
</tr>
<tr>
<td>4B/72s-4</td>
<td>67</td>
<td></td>
<td>12.2</td>
<td>18.3</td>
</tr>
<tr>
<td>4B/57x-4</td>
<td>85</td>
<td></td>
<td>8.0</td>
<td>9.0</td>
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<tr>
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<td></td>
<td>20.2</td>
<td>24.0</td>
</tr>
<tr>
<td>3D/49s-2</td>
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<td></td>
<td>11.4</td>
<td>16.0</td>
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<tr>
<td>3D/5s-1</td>
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<td></td>
<td>8.6</td>
<td>11.2</td>
</tr>
<tr>
<td>3D/49s-5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>80</td>
<td></td>
<td>12.2</td>
<td>14.5</td>
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<td>100</td>
<td></td>
<td>17.2</td>
<td>21.3</td>
</tr>
</tbody>
</table>

<sup>*</sup> Appeared to be PCR negative (segregants)
<sup>1</sup> s=seled, x=crossed to wild-type
<sup>2</sup> B. napus cv Topaz
particular importance when attempting to assign gene function in an amphidiploid plant such as *B. napus*.

I chose to use the antisense technique to attempt to determine the function of A9, a highly abundant (estimated at 0.2% of anther message at the microspore release stage of microsporogenesis) tapetal-specific transcript identified in *B. napus* (Scott *et al.*, 1991a). The high abundance of this message coupled with the independent identification of a similar transcript (LHM 7) in *Lilium* tapetal cells (Crossley, 1993) suggested that the A9 gene is present in a wide range of plants and may therefore have an important role in microsporogenesis.

Transformation of *B. napus* with a chimaeric gene encoding antisense RNA, resulted in the almost complete abolition of A9 gene expression in several plants. However, these plants exhibited normal male fertility indicating that under the conditions studied, the A9 gene is not required for pollen development and function. Downregulation of plant mRNAs without obvious phenotypic effect has been reported previously. Hall *et al.* (1993) inhibited the expression of a pectin esterase (PE) gene in tomato and Neuhaus *et al.* (1992) the expression of a β(1,3) glucanase in tobacco, both without major phenotypic effects. The first authors suggested that the observed phenotype was due to residual PE activity or due to another PE isoform. Neuhaus *et al.* (1992) proposed that β(1,3) glucanase in tobacco could be important in protection against pathogens and therefore not required in healthy plants. All three explanations could be considered in the present work. In the case of A9, it is possible that residual A9 expression in antisense plants may be sufficient to achieve full male fertility in the ideal conditions in which the plants were grown. It is also possible that other related proteins may functionally substitute for A9 in these plants. This idea is supported by the finding that the *B. napus* anther cDNA library (Scott *et al.*, 1991a) contains two cDNAs (A8 and C173) with a low but significant homology to the A9 cDNA. The corresponding transcripts of these cDNAs have a similar temporal pattern of expression as the A9 mRNA (Scott *et
al., 1991a and unpublished data) and the low level of nucleotide sequence similarity make it unlikely that these A9 related messages are downregulated in A9 antisense plants. Nonetheless, the significant sequence divergence of A9, A8 and C173 makes it unlikely that they are efficiently functionally interchangeable. It is also difficult to explain why A9 expression is so abundant if only a fraction of the normal A9 mRNA levels are sufficient for full male fertility and why A9 related genes are present in widely diverse plant species. An alternative explanation is suggested by the sequence homology of A9 to a family of proteins previously found only in seeds (Paul et al., 1992). Members of this group are seed storage proteins that generally have an unusual amino acid composition (for example they may be sulphur-rich), or they act as protease or alpha-amylase inhibitors. Kreis et al. (1985) recognised three regions of homology (A, B, C), shared by most members of this superfamily. The region B which is conserved greatly in the superfamily is also present in A9. This region carries a distinctive pattern of cysteine residues. It is possible that the members of the superfamily retain this pattern of cysteine residues to maintain a tertiary structure that is thermally and chemically stable and resistant to proteolysis. Such attributes may be of importance to A9 if it is secreted into the anther locule. The predicted A9 protein carries a hydrophobic N-terminal sequence which is likely to form a signal peptide (Paul et al. 1992). Thus, A9 might be secreted into the locule, as the tapetum is primarily a secretory tissue. Since the putative A9 protein does not possess an unusual amino acid content that would indicate a role as a storage protein it was suggested that A9 may be a defence protein that prevents pathogen or predator damage (Paul et al., 1992). Recently, Terras et al. (1992, 1993) reported that 2S albumins and nonspecific lipid transfer proteins extracted from Raphanus sativus and other Brassicaceae seeds possess antifungal activity. These proteins are related in sequence to A9 (see Chapter 7 for alignment). Thus it is possible that A9 may similarly possess antifungal activity. If A9 is a defence related protein it is not surprising that A9 expression can be abolished without any apparent phenotypic effect. Such an
explanation mirrors the results of downregulating the expression of a presumably defence-related tobacco β(1,3) glucanase gene (Neuhaus et al., 1992). Thoma et al. (1993) reported that a nonspecific lipid transfer protein from Arabidopsis was a cell wall protein and they suggested that it could be a defence-related protein.

In conclusion, although analysis of the transgenic plants expressing antisense A9 RNA has not yet shed light on the precise role of the A9 gene product, the results obtained have favoured the idea that A9 may be a defence-related gene. Further experiments such as challenging the A9 antisense plants with various pathogens and the localisation of the A9 protein within the anther may further clarify the role of the A9 gene product.

6.4 Summary
An antisense approach was used to attempt to determine the function of the highly abundant, tapetum-specific A9 transcript in microsporogenesis. A B. napus A9 cDNA clone was linked in sense and antisense orientations to the A. thaliana A9 promoter and CaMV 3Ssx2 promoter, and the resulting chimaeric genes introduced into tobacco, A. thaliana and B. napus. Both sense and antisense B. napus A9 RNA was detected in tobacco, although sense RNA levels were significantly higher than antisense. A9 sense and antisense transformed A. thaliana plants showed normal fertility level. A high proportion of the offspring of B. napus antisense A9 plants had very low or undetectable levels of A9 mRNA. However, these plants set seed and had pollen of normal or near normal viability. Therefore, under the conditions studied, the A9 protein appears not to be essential for male fertility in B. napus.
CHAPTER 7

DISCUSSION

7.1 General Discussion
The main aim of work described in the thesis was to study anther development of *B. napus* using an antisense RNA approach. Attempts were also made to use *A. thaliana* as a model plant because of its close phylogenetic relation with *B. napus* and reportedly more efficient transformation system. Initially, transformation of *A. thaliana* and *B. napus* with *A. tumefaciens* was studied in an attempt to improve transformation efficiencies to a level that would allow the generation of large numbers of independent transformants. This was especially successful for *A. thaliana* (Chapter 3) since the outcome was the development of an efficient transformation method based on immature and mature cotyledon explants. A certain amount of success was also achieved with *B. napus* via a similar route (Chapter 4). A novel finding was regeneration via embryogenesis. After these preliminary experiments, anther development of *B. napus* was studied. For this part of the project, two anther-specific cDNAs, A1 (Chapter 5)
and A9 (Chapter 6), were chosen for antisense RNA experiments. However, the A1 cDNA was excluded from the antisense RNA experiments before any transgenic plants were generated. There were two reasons for this. First, since the original A1 clone represented only a 3'-end fragment of the transcript, attempts were made to obtain a full length clone, but these were unsuccessful. Second, several unsuccessful attempts were also made to obtain an A1 gene from an Arabidopsis genomic library; consequently, the A1 promoter was not available to drive A1 antisense RNA expression in transgenic plants. However, one positive outcome was that the A1 peptide sequence was shown to share amino acid sequence similarity with chalcone synthase (CHS) and stilbene synthase (STS) enzymes.

In contrast to A1, a comprehensive antisense RNA experiment was successfully conducted with the A9 cDNA. The cDNA, which is tapetum-specific, was fused to the Arabidopsis A9 promoter in the sense and antisense orientation and used to transform A. thaliana and B. napus. However, little work was undertaken with the A. thaliana transgenics when it became apparent that the very small size of the flower at the stages of development when the A9 gene is active presented extreme technical difficulties to the analysis. Consequently, the bulk of the analysis was carried out in B. napus.

7.2 Transformation of B. napus and A. thaliana

B. napus and A. thaliana were studied to improve Agrobacterium-mediated transformation efficiencies. Both species are naturally susceptible to infection by A. tumefaciens. Initially, adventitious shoot regeneration capacities from both species were investigated using various plant growth regulators because efficient transformation methods require the expression of good regeneration capacity. Cotyledonary explants from both species appeared to be the most suitable explants for organogenesis and also Agrobacterium-mediated transformation. Significantly, cotyledonary explants produced shoots at the cut surface via callus formation, which is very important for A. tumefaciens.
infection. This finding has been reported by other researchers (Moloney et al., 1989; Sharma et al., 1991). This cut surface is an ideal target for *A. tumefaciens* because the cells undergoing organogenesis are readily accessible to the agrobacteria. Also, cotyledonary explants produced a high frequency of shoot regeneration within 4-5 weeks which could reduce the possibility of somaclonal variation. Moreover, the use of cotyledonary explants is straightforward, since the starting material is grown in sterile conditions and is ready for use 3-5 days after sowing. Therefore, it is possible to establish both good regeneration and transformation systems without any shortage of plant material.

An efficient method for *Agrobacterium*-mediated transformation of *A. thaliana* has been developed based on the use of cotyledons. There are some advantages of this method over other published transformation methods (see Chapter 3). Firstly, rapid transgenic shoot regeneration was achieved from cotyledon explants which, due to very short callusing phase, could reduce the possibility of somaclonal variation. It is also desirable to obtain transformed plants as rapidly as possible for convenience. Secondly, a high frequency (3-4 transformed shoots/explant) of transgenic plants was obtained. However, working with *Arabidopsis* in tissue culture was difficult due to very small sizes of explants.

The transformation efficiency of *B. napus* was inferior to *Arabidopsis*, although prolific shoot regeneration was obtained from cotyledonary explants. With the exception of Moloney et al., (1989), all other reports revealed low frequency of transformation from *B. napus*. Moreover, the method of Moloney has not been repeated successfully in terms of achieving high levels of transformation. As described in Chapter 4 high levels of shoot regeneration were obtained using immature and mature cotyledons. Several modes of organogenesis were observed, including direct and indirect embryogenesis. Transgenic plants were successfully generated by using mature cotyledonary explants from *B. napus* cv. Cobra.
7.3 Study on Anther Development of *B. napus*

Two cDNA clones, A1 and A9, isolated from a *B. napus* cDNA library constructed from anthers of 1.2-1.8 mm buds (Scott *et al.*, 1991a) were investigated to study anther development. A1 cDNA which is a high abundance clone and represents anther specific transcripts has been sequenced and deduced amino acid sequence has been obtained. The A1 cDNA clone showed significant levels of nucleotide sequence similarity to C19, which was isolated from same cDNA library and represents a low abundance transcript (Hodge *et al.*, 1992). Although the A1 cDNA did not represent a full-length mRNA the amino acid sequence showed similarity to published chalcone synthase (CHS) and stilbene synthase (STS) sequences. The A1 peptide also showed significant similarity to the protein encoded by the BA42 transcript, an anther-specific mRNA isolated from *B. napus* (Shen and Hsu, 1992). The degree of similarity between BA42 and A1 suggest that they may be the result of a relatively recent gene duplication event. Thus, the A1, C19 and BA42 represent a small family of *B. napus* anther-specific genes which encode proteins showing similarity to CHS and STS sequences. Shen and Hsu (1992) reported that the BA42 message is present in the tapetum and vascular tissue of immature anthers and also in young microspores. The localisation of the A1 and C19 messages was not determined in this work.

Constructs designed to express sense/antisense A1 RNA driven by the tapetum-specific A9 and CaMV 35S promoters were generated and used to transform tobacco to determine if these constructs express *B. napus* A1 RNA. Both constructs expressed *B. napus* A1 RNA in transformed plants although the levels of antisense RNA were much lower than sense levels. These sense/antisense constructs were not used to transform *B. napus* or *A. thaliana*, since the A1 promoter was not available and the A1 cDNA was not full length. In contrast to A1, there was more information about the expression pattern of the A9 gene: the spatial expression pattern was demonstrated by in situ hybridization (Scott *et al.*, 1991a); the A9 promoter had been isolated from
Arabidopsis (Paul et al., 1992) and promoter-GUS and barnase genes used to accurately determine the temporal expression pattern and confirm the spatial pattern (Paul et al., 1992). Thus, A9 sense/antisense RNA constructs driven by the A9 promoter were used to transform A. thaliana and B. napus (see Chapter 6) to understand the role of A9 protein during anther development. Initially, sense and antisense RNA constructs were introduced into tobacco to determine if these constructs express B. napus A9 RNA. It was demonstrated that both sense and antisense constructs express A9 RNA in transformed tobacco. These constructs were then used to transform A. thaliana in order to produce informative mutants in a relatively short time. All sense and antisense transformed A. thaliana plants showed normal fertility level and set seed normally. However, transformation work with A. thaliana was abandoned as it was difficult to work on flower development due to its small size. Consequently, B. napus which is much larger than A. thaliana, was studied in order to achieve greater resolution of any mutant phenotypes. Four antisense and a single sense transformed plants were produced, thus only antisense transformed plants were studied further. The original transformed plants showed low levels of fertility. Since this could have been due to tissue culture effects, T2 and T3 generation plants checked by PCR were investigated by northern blot analysis and microscopy of pollen development. Within these families, a high proportion of individuals carrying the antisense gene plants showed very low or undetectable levels of A9 mRNA. However, the reduction in pollen viability was not correlated with downregulation of A9 message levels. Some A9 antisense transformed plants and an untransformed plant showed reduced pollen viability but set seed normally. As mentioned previously, it is possible that the reduced pollen viability observed in some plants may be due to a mutation generated during tissue culture that does not segregate with a T-DNA insertion.

A striking outcome of things work is the observation that despite a severe reduction in A9 mRNA levels in some antisense transformants, the plants showed normal male fertility. Therefore, under the conditions studied, the A9
protein appears not to be essential for male fertility in *B. napus*. Therefore, what is the role of the A9 transcript which is both highly abundant and, as judged by barnase fusions which led only to ablation of the tapetum, completely tapetum-specific? Transcripts that encode polypeptides with features in common to A9 have been reported in *Antirrhinum majus* (Nacken et al., 1991), *Lilium* (Crossley, 1993) and *Lycopersicon esculentum* (Chen and Smith, 1993) demonstrating that the A9 gene homologues exist in a broad range of species and suggesting that the gene product may play an important role in microsporogenesis (Figure 7.1, Figure 7.2). Although the level of overall homology is low to any sequence in the databases, A9 contains structural motif present in a diverse group of seed proteins (Figure 7.3). Kreis et al. (1985) identified three regions of homology (A, B, C) in this superfamily and the region with greatest conservation (region B) is also present in A9. This region contains a distinctive pattern of cysteine residues (i.e. "CC...C-C"). It has been reported that cysteines are involved in intramolecular disulphide bridges (Maeda et al., 1983; Lille and inglis, 1986). Recently, it was reported that 2S albumins and nonspecific lipid transfer proteins extracted from the seed coat of *Raphanus sativus* and other Brassicaceae showed antifungal activity (Terras et al., 1992; 1993). These experiments involved suspending fungal spores in seed protein solutions, and incubating for an appropriate time period. Growth of the microorganism was then evaluated by measuring the absorbance of the culture. Various of the fungi were inhibited by the seed proteins. The amino acid sequence of A9 is related to these proteins; therefore it is possible that A9 may have similar antifungal activity. If A9 is a defence related protein, it is not surprising that A9 expression can be abolished without any apparent phenotypic effect. However, A9 and other similar proteins must be effectively maintained by "Natural Selection" since genes closely-related by nucleotide sequence are found in *A. thaliana* and *B. napus*, and putative peptides containing the structural motif are extremely widely dispersed throughout the higher plants. Therefore, the gene must be essential or advantages under
Figure 7.1
Alignment of the predicted amino-acid sequences of A9 with other anther-specific sequences. Sequences were aligned using the program CLUSTAL. Asterisks identify residues shared by all sequences, dots indicate conservative substitutions. Le, *L. esculentum* (Chen and Smith, 1993); Am, *A. majus* (Nacken et al., 1991); Lily (Crossley, 1993); At, *A. thaliana*; Bn, *B. napus* (Paul et al., 1992).
Le

MASVKSFFSSSSSFSISLILLLLVIVLQSQVIQECQPQSCCTASLTLGLNVCAFLPFLPGSFP

Am fill

MAMKSYVPLNLTVLVAQSLTQSEAQTCSASLALNACAPFVLGA

Bn A8

MVSISSSSSSSTMVVMVAVLVAVTVDGQSCNYLSGLNVCOHPFVPGAD

Lily

RGLSQNCSSAAIGELMTGLFPYVPGSN

At A9

MVLSKSLAAILVAMFLATGPTVLGQCRDSGLNVQVCAPLFSQAV

Bn A9

MEFLASFTTILFVMFLAMSALETVMVRAGQCLUNLSMQVCAPLPGAV

** . . . .

Le

TA---STECNAVQISNEDCNMTRIAAQIPAGCNLPFLCSAN

Am fill

TT-PSSDCTALQSQVDMELCMTLRIASRVPAQCNLPFLCSAN

Bn A8

TTNPSAECCHALAEAPSDCTCHRTFRIASRLPTRTCHPTLSCN

Lily

GA---PQCCSAKAVNHGCLCEITINISSLPLDNLPLPAVNC

At A9

NPAANSNDCAALQCNIKCIULRRAATLTSLCNPSSDCGMLHIKLKPLDFFYKLMH

Bn A9

NPAPNSNCIALQATNFCICNALRAATTTTCNLSLDCQITI

** . . . . * . . . . . .
Figure 7.2
Dendogram based on the alignment in Figure 7.1. Le, *L. esculentum* (Chen and Smith, 1993); Am, *A. majus* (Nacken et al., 1991); Lily (Crossley, 1993); At, *A. thaliana*; Bn, *B. napus* (Paul et al., 1992).
% similarity between amino-acid sequences
Figure 7.3
Alignment of the predicted amino-acid sequences of A9 with lipid transfer proteins (LTP). Sequences were aligned using the program CLUSTAL. Asterisks identify residues shared by all sequences, dots indicate conservative substitutions. At, A. thaliana (Botella et al., unpublished); Zm, Zea mays (Arondel et al., unpublished); Nt, N. tabacum (Fleming et al., 1992); Hv, Hordeum vulgare (Linnestad et al., 1991); At, A. thaliana; Bn, B. napus (Paul et al., 1992).
AtLTP  KTTSLEFGEVCMALLHLCGVRNNTSNAALSQGVSNLAAACIGYVIQGVIAPP---CC
ZmLTP  SCOQVASAIAPCISYARGQGSQPSAG---CC
NtLTP  MEIAQKIACFVVLCHVAAACAAITCQGVTSHLAPCLALHNR---TGFLG---CC
HvLTP  NARAQVLAMGAAALVLMTAAPRAAVNLNQVYDSSKCPCTVQQ-GFGPSGE---CC
At A9  MVSLSLAAILVANFLATGPIFTVLAQRCREDLSNQQVCAPLLPKGAVNPAANSNC
Bn A9  MEFLKSLFTTLFVNFLASALETVNVRAGQLCLNLSSRKVCAPLVPGAVNPAANSNC

* * * * *

AtLTP  SOVKNLNSIAKTTPDRQQACNCIQQGAASAALGGLNAGRAAGIPACGVRNHSYKTSTSC
ZmLTP  SOVRSLNIAARTTADERAACNCNLNSAAGV-SGLNAQSAASIPSKOVSIPTTISTSTDC
NtLTP  GVQALVNSAARTTDERAACTCLKSAAGA1-QGSLNGRAFGPINPIKIPSTSTDC
HvLTP  NVVRDLNQAOSSQRQTVCMCLKIGAEG1-HNNSNAASIPSKCNVYPIPSSTSDC
At A9  AAALQA---------TNKDCNLNRRAAT----------TLTSLCNL---------PSFDC
Bn A9  IALQAA---------TNKDCNLNRRAAT----------TTPTICNLC---------PSLD

* * * * *

AtLTP  KTVSDELATVR
ZmLTP  SREYGRNHSAD
NtLTP  S
HvLTP  S
At A9  NRMIHLKAPLLLDFYKLFWQ
Bn A9  GITIEXIT
certain conditions. Although the location of the A9 protein is not known, the A9 transcript encodes a potential signal peptide which presumably targets the A9 protein to the endoplasmic reticulum (ER), from where it presumably either secreted into the lumen of the locule or sequestered within a tapetal storage vesicle of some kind. Some of the seed storage proteins are intracellular whilst some others are located into cell wall. Although it is not possible to say where the A9 might be located the pollen coat proteins could be isolated (Doughty et al., 1993) from wild-type and antisense plants to see if there is a difference between them. The absence of a protein of the predicted size of A9 within the wall proteins would strongly suggest that the A9 protein is localised in the exine and would support an antifungal role. However, one puzzling fact is that A9 gene expression commences at the onset of meiosis, well before the pollen grains form. Possibly, A9 protein accumulates in the tapetum before being secreted into the locule. Another question concerning A9 biology is why, given the non-essential nature of the gene product, the expression of the gene is subject to such tight transcriptional regulation? One possibility is that this is a feature of genes that carryout tapetum-specific functions, since promoter fragments from TA29 (Mariani et al., 1990), A3 (Scott et al., 1991b) and A9 (Paul et al., 1992) have all been demonstrated to be tapetum-specific by barnase fusions.

The apparent relatedness of A9 to various seed proteins is in accord with the generally accepted view that, as a consequence of ancestry and functional homologies (both are propagules) there is an analogy between pollen and seeds. Other examples of similarities at the molecule level include the sequestration of lipids into oil bodies in both pollen and seeds. These oil bodies are stabilized by specific proteins, oleosins, which are encoded by pollen and seed specific genes that are related by sequence (Roberts et al., 1993b).

To determine the roles of genes for male fertility requires large scale antisense experiments. The first step is to make constructs from a large number of anther-specific cDNAs. Spatial expression of these cDNAs in anther
development could be determined by northern gel blots or in situ hybridization. Scott et al., (1991a) hybridized anther-specific cDNAs (A9 and #17) with bud RNA samples derived from both male sterile which has no pollen grains and male fertile plants. They showed that A9 hybridized to both RNA samples whilst #17 hybridization was restricted to the male fertile RNA samples. A critical consideration is that the temporal and spatial expression pattern of the promoter chosen to drive the antisense constructs should closely match those of the target RNA. In some cases, the CaMV 35S may suffice, but a specific promoter, perhaps derived from the target gene, is preferable. The second step is to transform the target species with antisense constructs. For large scale experiments, the transformation frequency should be sufficiently high to generate many independent transformants. Therefore, initial antisense experiments could be carried out with tobacco which is easy to transform and to work on flower development because of its large size. Alternatively, the transformation frequencies in crop plants (e.g. B. napus) should be improved and used routinely. When the above conditions are fulfilled, large scale antisense experiments will become more feasible.

7.4 Future Work

7.4.1 Agrobacterium -Mediated Transformation

Despite the fact that cotyledonary explants of B. napus produced good adventitious shoot regeneration, transformation efficiencies were poor relatively to those routinely obtained in species like tobacco (see Chapter 4). Cotyledons apparently have a high potential for Agrobacterium -mediated transformation, but for some reason transformation frequencies are low. Initial experiments showed that the A. tumefaciens wild-type oncogenic strain C58 was the most appropriate for B. napus. This strain induced 20% tumour formation. However, the frequency of transformation was reduced to 5% when disarmed A. tumefaciens strain (pGV3850::pBI121) was used to transform B. napus. This suggests a problem exists with the expression vector, since pGV3850 has same
chromosomal background as C58. In this case, *Agrobacterium* infects the explants successfully but neither the NPT II or GUS genes express to detectable levels within the cells of the explants. Therefore, the routine high frequency transformation of *B. napus* may require a suitable plasmid vector constructed specifically for *B. napus* transformation. Another possible explanation for the low transformation frequency concerns the regeneration of transformed cells. After co-cultivation, callus sectioning of cotyledonary explants showed that a high frequency of the explants had evidence of transient GUS expression within the callus. However, these calli tended to produce untransformed shoots which were most probably initiated from untransformed cells. It is possible that uninfected cells undergo cell division or cell enlargement much easier than infected cells. Hence, uninfected cells regenerate preferentially. In this case, co-cultivation could be a crucial stage for efficient *Agrobacterium* infection of the explants. The following factors appear to be the most important: quantity and quality of the bacterial culture used for co-cultivation; infection time; accessibility of the explants for *Agrobacterium*; co-cultivation time and conditions.

Immature cotyledonal explants of *B. napus* showed particularly high levels of prolific shoot regeneration and somatic embryogenesis (see Chapter 4) which could be very useful for *Agrobacterium*-mediated transformation. In some cases, adventitious shoots initiated from subepidermal tissues of the immature cotyledons which may not be accessible to *Agrobacterium* infection. However, it is possible to induce callus formation by changing the concentration of the plant growth regulators since immature cotyledons showed very good response to various growth regulators. It is obvious that immature cotyledons have much more potential for organogenesis than mature cotyledons. Therefore, transformation experiments with immature cotyledonal explants should be carried out to achieve higher transformation efficiency. The results presented in Chapter 4 could give some important information for further work.
7.4.2 Functions of A1 and A9 Proteins

The A1 cDNA isolated from the *B. napus* cDNA library is a high abundance clone and expressed during the tetrad and microspore release stages of microsporogenesis (Scott *et al.*, 1991a). However, the spatial expression of A1 needs to be investigated either by *in situ* hybridization or by A1 promoter-GUS or barnase fusion experiments. The predicted amino acid sequence of A1, which is not full-length, showed similarity to chalcone synthase and stilbene synthase enzymes (see Chapter 5). From the sequence data it is not possible to predict if A1 encodes a CHS or STS type activity or whether it represents a new class of enzyme. Two techniques could be used to investigate the function of A1 protein: 1) antisense approach, 2) overexpression of A1 in *E. coli* and enzyme assays. Both of these approaches, and the determination of the spatial expression pattern require a full-length A1 transcript or genomic clone. Therefore a *B. napus* anther cDNA library and *Arabidopsis* genomic library were re-screened. Nevertheless, several attempts to isolate full-length A1 cDNA or A1 gene failed. In Leicester University, Mike Roberts constructed a new *Arabidopsis* genomic library which could be screened to isolate the A1 gene. The sense and antisense RNA driven by tapetum-specific A9 promoter and CaMV 35 promoter have already been constructed and used to transform tobacco. BA42 which showed significant similarity to A1 at the amino acid level expresses in the tapetum and vascular tissue of immature anthers and also in young microspores (Shen and Hsu, 1992). It is also predicted that A1 message may be present in anthers in different cell types from the A9 transcript. Therefore, the A9 promoter is not suitable to drive A1 sense and antisense RNAs in order to downregulate the A1 mRNA of *B. napus*. Consequently, the A1 promoter should be isolated from the A1 gene of *Arabidopsis* and used to drive A1 sense and antisense RNA constructs, since the A1 promoter will express in both the correct tissues and at the correct time. Although antisense RNAs in transformed plants have led to the identification of proteins (Hamilton *et al.*, 1990; Bird *et al.*, 1991), in some cases plant mRNAs have been downregulated
without obvious phenotypic effect as happened with A9 (Neuhaus et al., 1992; Hall et al., 1993). It can be speculated that A1 is probably a defence related protein so that downregulation of A1 mRNA may not lead to understand the role of the A1 protein in normal conditions. As mentioned previously, the A1 peptide showed similarity to chalcone synthase which is the key enzyme in the flavonoid biosynthesis, and stilbene synthase. Flavonoids are involved in many plant processes including pigmentation, defence and U.V. protection and stilbenes are present in a limited number of plant species and their presence is usually associated with stress or fungal attack. Hence, it is possible that A1 protein is involved in similar functions in anther development. If antisense approach fails to show phenotype, A1 gene could be overexpressed in *E. coli* to purify A1 protein which require antibody raising. As far as I am concerned, A1 probably has a very important function in flower development and which could be exploited in practical ways.

At the beginning, there were more data about A9 which is a highly abundant, anther-specific transcript in microsporogenesis (see Chapter 6). The A9 gene and promoter sequence were isolated from *Arabidopsis* genomic library, and also in situ hybridization and A9 promoter-GUS and barnase fusions demonstrated that A9 transcript is present only in the tapetum (Paul et al., 1992). Therefore, it appeared that the A9 gene should have a very important role in male fertility of *B. napus*. Kaul et al., (1988) reported that natural male sterility is often linked to tapetal malfunction. The tapetum-specific promoter (TA29) from tobacco used to drive barnase resulted in destruction of the tapetum and, as expected, in male sterility due to a failure to produce pollen grains (Mariani et al., 1990). It was also expected that A9 protein could have similar function, thus downregulation of A9 mRNA could cause male sterility. Therefore, A9 sense and antisense RNA constructs driven by A9 promoter were introduced into *B. napus* to inhibit A9 gene expression in anther development. Transformed plants showed very low or undetectable levels of A9 mRNA but they had pollen of normal and near normal viability, and set seed normally. Therefore, contrary to
expectations, the A9 protein appeared not to be essential for male fertility in *B. napus*. Furthermore, these antisense transformed plants did not show any phenotype other than male fertility under the conditions studied. The possible reasons why there was no obvious phenotype observed, were discussed in Chapter 6. Terras *et al.* (1992; 1993) reported that 2S albumins and nonspecific lipid transfer proteins which are related to A9 protein in sequence, showed antifungal activity. These findings strongly suggest that A9 could be a defence-related gene so that it is normal that A9 expression can be abolished with no phenotypic effect. To test A9 for antifungal activity, A9 antisense and wild-type *B. napus* plants could be challenged with various pathogens to see if there is a difference between transformed and wild-type plants. Alternatively, as reported by Terras *et al.*, (1992; 1993), spores from various fungal strains could be grown in A9 protein once the A9 protein has been overexpressed and semi-purified. However, proteins synthesized in bacteria could fold incorrectly or inefficiently and therefore show poor biological activity. A further possibility is to make protein extracts from A9 antisense and wild-type pollen and use these in bioassays. These techniques have some difficulties, but they are the most suitable methods with which to understand the function of the A9 protein in *B. napus*. Recently, a similar transcript (LHM 7) has been identified in *Lilium* (Crossley, 1993). This report suggested that the A9 gene is present in a wide range of plant species and therefore understanding of the role of the A9 gene would find a broad range of application. If the A9 protein has a antipathogenic activity it will be very useful in the production of pathogen resistant crop plants.
APPENDICES

Appendix I: Bacterial Media

Liquid Media

NB (nutrient broth) was purchased as a pre-made powder from Difco Laboratories and dissolved in water and sterilized at 121 °C for 20 minutes.

**NB**

- **LB (Luria broth): per litre**
  - Bacto-tryptone 10 g
  - Bacto-Yeast extract 5 g
  - NaCl 10 g

Adjust the pH 7.0 with 1M NaOH and sterilize.

- **NZY: per litre**
  - NZ Amine* 10 g
  - Bacto-Yeast extract 5 g
  - NaCl 5 g
  - Caesin amino acids 1 g
  - MgSO₄ 2 g

(*NZ Amine is type 2 caesin hydrolysate)
Adjust the pH 7.5 with 1M NaOH and sterilized.

- **2xYT: per litre**
  - Bacto-tryptone 16 g
  - Bacto-Yeast extract 10 g
  - NaCl 5 g

Adjust the pH 7.0 with 1M NaOH and sterilized.

Semi-Solid Media

All media was solidified by the addition of Bacto-Agar to 1.5%.
Appendix II: Plant Tissue Culture Medium

Murashige and Skoog Salt, 1962

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<tr>
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Above components are marketed in preweighed packets by Flow Lab. Ltd.
Hoagland's Solution

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Appendix III: Molecular Biology Buffers and Solutions

TE : 10 mM Tris-Cl pH 7.5, 1 mM ethylenediamine tetracetic acid (EDTA)

10x TAE : 0.4 M Tris-acetate pH 8.0, 10 mM EDTA

10x TCM : 0.1 M Tris-Cl pH 7.5, 0.1 M CaCl₂, 0.1 M MgCl₂

10x TBE : 121 g Tris base, 7.4 g EDTA, 53.4 g boric acid per litre distilled water

20 x SSC : 3 M NaCl, 0.3 M sodium citrate

20 x SSPE : 3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA, pH 7.7

50 x Denhardts : 1% BSA, 1% Ficoll 400, 1% PVP

Phenol/Chloroform : Phenol:Chloroform:isoamyl Alcohol, 25:24:1 plus 0.1% 8-hydroxyquinoline, equilibrated and overlayed with 0.1 M Tris-HCl pH 8.0.
SM Buffer: 0.5 M Tris-HCl pH 7.5, 0.1% gelatin, 0.1 M NaCl, 15 mM MgSO₄

2x CTAB: 0.1 M Tris-Cl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2.8 μl/ml β-mercaptoethanol, 2% CTAB

10% CTAB: 10% CTAB, 0.7 M NaCl

RNA extraction buffer: 0.1 M LiCl, 100 mM Tris-HCl, 10 mM EDTA, 1% SDS

10x MOPS Buffer: 0.2 M 3-[N-morpholino]-propane sulphonic acid, 0.05 M sodium acetate pH 7.0, 0.01 M EDTA

RNA sample Buffer: 0.75 ml formamide, 0.15 ml 10x MOPS, 0.24 ml formaldehyde, 0.1 ml RNase-free H₂O, 0.1 ml glycerol, 0.08 ml bromophenol blue

RNA extraction buffer: 0.1 M LiCl, 100 mM Tris-HCl, 10 mM EDTA, 1% SDS

Depurinating solution: 0.25 M hydrochloric acid

Denaturing solution: 0.5 M NaOH, 1.5 M NaCl

Neutralizing solution: 3.0 M NaCl, 0.5 M Tris-Cl pH 7.4

DNA hybridization solution: 3x SSC, 5x Denhardts, 0.5% SDS, 0.2 mg/ml sheared herring sperm DNA

RNA hybridization solution: 50% formamide, 6x SSPE, 5x Denhardts, 0.4% SDS, 6% polyethyleneglycol (PEG 6000), 0.2 mg/ml sheared herring sperm DNA

Oligolabelling Buffer: Combine 625 μl 2 M Tris-HCL pH 8.0, 25 μl 5 M MgCl₂, 350 μl dH₂O, 18 μl β-mercaptoethanol, 5 μl each of 3 mM dATP, dGTP, dCTP

Oligolabelling stop solution: 20 mM NaCl, 20 mM Tris-Cl pH 7.4, 2 mM EDTA, 0.25% SDS

Wash solution A: 3x SSC, 0.5% SDS

Wash solution B: 0.5x SSC, 0.5% SDS

5x Ligation Buffer: 0.25 M Tris-HCL pH 7.6, 50 mM MgCl₂, 50 mM dithiothreitol, 250 μg/ml BSA, 5 mM ATP

CIP buffer: 50 mM Tris-HCL pH 8.0, 0.1 mM EDTA, 20 mM ZnCl₂, 0.5 mM MgCl₂
Gel loading buffer: 0.25% bromophenol blue, 10% Ficoll (type 400), 0.1 M Na₂EDTA, 2% SDS, 1x TAE

Lysis buffer: 25 mM Tris-Cl pH 8.0, 10 mM EDTA, 0.5 M glucose

Alkaline SDS: 0.2 M NaOH, 1% SDS

DNA extraction buffer (PCR): 200 mM Tris-Cl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS

10x PCR buffer: 67 mM Tris-Cl pH 8.8, 16.7 mM ammonium sulphate, 6.7 mM MgCl₂, 10 mM β-mercaptoethanol, 6.7 μl EDTA pH 8.0, 1.5 mM each dNTP, 170 μg/ml BSA

5x Sequenase buffer: 200 mM Tris-Cl pH 7.5, 100 mM MgCl₂, 250 mM NaCl

Sequenase stop solution: 95% formamide, 20 mM EDTA, 0.05 bromophenol blue, 0.05% xylene cyanol FF

GUS extraction buffer: 50 mM NaPO₄ pH 7.0, 10 mM EDTA, 0.1% Triton-X 100, 0.1% Sarkosyl, 10 mM β-mercaptoethanol

Bradford’s solution: 600 mg/ml Serva blue G-250 stain in 2% perchloric acid
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