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Cloning strategies for the isolation of an α-amylase inhibitor/endochitinase gene from the seeds of *Coix lachryma-jobi.*

Donna Fairweather

A thesis submitted to the Department of Biological Sciences, University of Durham in accordance with the requirements for the degree of Doctor of Philosophy

Department of Biological Sciences
University of Durham

February 1993

22 SEP 1994

CLONING STRATEGIES FOR THE ISOLATION OF AN α-AMYLASE INHIBITOR/ENDOCHITINASE GENE FROM THE SEEDS OF *COIX LACHRYMA-JOBI*

DONNA FAIRWEATHER

PhD 1993

ABSTRACT

The aim of this work was to isolate the gene encoding a bifunctional α-amylase inhibitor/endochitinase protein from the seeds of *Coix lachryma-jobi*, a tropical cereal. Prior to this study, it had been demonstrated that this bifunctional protein had anti-insect and possibly anti-fungal properties. Consequently the gene could potentially be used to confer insect and fungal resistance in transgenic plants.

A multifunctional approach was undertaken to isolate the α-amylase inhibitor/endochitinase cDNA and genomic sequences, involving three main strategies.

Immunoscreening a *Coix* cDNA expression library with antibodies raised against a wheat germ endochitinase protein resulted in the isolation of three immunopositive clones. These cDNA's, were sequenced and one characterised as a seed storage protein, named α-coixin. Despite extensive searches of the appropriate databases, the function of the other two are as yet unknown.

Another strategy was the production of polyclonal antibodies, raised against a glutathione S-transferase-α-amylase inhibitor fusion peptide. It was envisaged that these antibodies could be used to isolate the gene of interest following immunoscreening of the *Coix* cDNA expression library. Polyclonal antibodies were successfully elicited against the glutathione S-transferase moiety, but could not detect the α-amylase inhibitor protein when assayed.

Using the polymerase chain reaction, amplification of the α-amylase inhibitor coding sequence was attempted from *Coix* genomic DNA, cDNA and a *Coix* seed cDNA library. PCR product were successfully amplified from genomic DNA and the cDNA library. Further characterisation of these product revealed that they were a result of non specific amplifications.

Further work required to isolate the α-amylase inhibitor gene is discussed.

DECLARATION

No part of this thesis has been previously submitted for a degree at this or any other university. All the material is my own original work except where otherwise stated.

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ABBREVIATIONS

A_{260}	Absorbance at 260nm
A_{280}	Absorbance at 280nm
bp	base pairs
BSA	bovine serum albumin
cpm	counts per minute
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ddNTP	dideoxynucleoside triphosphate
DMF	dimethyl formamide
DMSO	dimethylsulphoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic
EtOH	ethanol
IPTG	isopropylthiogalactoside
kb	kilobase pairs
kDa	kilodaltons
Da	daltons
MOPS	3-[N-Morpholino]propanesulfonic acid
OD_{600}	optical density (absorbance) at a wavelength of 600nm
PEG	polyethylene glycol
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
TE	10mM Tris pH 8.0, 1mM EDTA buffer
TEMED	N,N,N',N'-tetramethlethylenediamine
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

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CHAPTER 1

1.1 General introduction

In 1990 the number of starving people was estimated at a staggering 700 million, this number is increasing daily. Latin America has some 70 million hungry; Africa has double that, with 140 million; and South Asia, including India, 350 million (Walgate, 1990). Traditional agricultural technology can no longer keep up with increased demand for food from a rising population, total yields seem to be reaching their peak. Rice, for example, provides the total calorie intake for 2 billion people, and in some areas of Asia it provides 70% of the protein in the diet. Production of rice must grow by nearly half by the year 2000 to meet the worlds demand. The long term solution to the food supply problem can only be fewer people, but this appears to be an impossible goal in the short or even medium term, one of the possible solutions is more food.

Plants alone cannot provide all the amino acids required for a healthy balanced diet, however in combination with animal foods, plants are a valuable nutritional source worldwide. As more total protein per acre land is obtained from plants, compared to animals, man will have to depend increasingly on plant foods to satisfy this increase in food demand.

Recent estimations fix the loss due to pests, weeds and diseases of the total potential crop production as 37%, damage by insects accounting for 13% (Gatehouse and Hilder, 1988). Increasing the efficiency of plant resistance mechanisms against pathogen attack would substantially increase the present crop yields. At present the most conventional methods of pest control involve breeding resistance into plants and intensive application of agrochemicals, in the form of pesticides, herbicides and fertilisers. Chemical control is expensive, world spending on pesticides has reached US$16 billion a year. The global pesticide market in 1987 was estimated at approximately U.S.$3,812 million dollors, to control pests of the worlds three major crops; cotton, maize and rice (Fischoff *et al.*, 1987). This high cost of pest control, along with the enviromental hazards often associated with chemical protection, has led to a considerable development in plant genetic engineering as a method of producing crop plants with enhanced resistance to insect, fungal and microbial pathogens.

Crop improvement by biotechnology is seen by many as the key to solving the worlds food problem. By exploiting biotechnology and genetic engineering, increased food production could come from finding and using new genes for increasing the resistance of high yielding modern crop varieties to pest and diseases, and extending their ability to thrive in harsher conditions.

1.2 Genetic engineering for resistance

Plant breeders have contributed to more than half of the 5-10 fold increase in agricultural yields over the past 50 years; the rest has come from improved agricultural practise and machinery, fertilisers, weedkillers, and pesticides.

Genetic improvements have been achieved through several plant breeding techniques such as, exploiting hybrid vigour and selecting for high yielding and pest resistant strains. However the transfer of these traits by sexual crossing is often laborious, extremely time consuming and is constrained by species determined incompatibility. Plant breeders have to put their plants through repeated rounds of interbreeding before they can produce useful breeding lines that contain the genes they want, and that will transmit them in a predictable manner. For example the three most important characteristics of European sugar-beet, high sugar yield, resistance to "bolting", and its production of a single seeded fruit took 25 years to select.

With the advent of genetic engineering came techniques which enabled scientists to isolate genes encoding desirable traits from numerous organisms. Transformation of several plants has been achieved from a donor which is sexually incompatible following the introduction of foreign genes or altered genes under the control of strong promoters by *Agrobacterium tumefaciens* gene transfer. Early transformations were confined mostly to dicotyledenous plants such as tobacco, petunia, potato and tomato with considerably fewer monocotyledonous plants being succesfully transformed. However with the development of new gene delivery techniques such as transformation of protoplasts with DNA using electroporation (Shillito and Saul, 1988) and microprojectile bombardment (Klein *et al.*, 1988) increased efficiency in the transformation of monocotyledons, particularly the cereal crops is envisaged. Additionally, the development of efficient tissue culture regeneration methods via somatic embryogenesis has improved the transformation efficiencies of monocots such as wheat (Kartha, 1992).

Scientist are continually searching for genes which encode for desirable traits for gene transfer. Many of the important traits in agriculture are controlled by the actions of multiple genes. Previous strategies to analyse the numbers and locations of these genes have relied upon statistical inference from data obtained from segregating populations. The use of molecular markers, in particular RFLP's has provided a new tool that has allowed the systematic assay of individual chromosomal regions for the presence of genes with major effects upon phenotypic variation. For example, studies to date in corn (Helentjaris *et al.*, 1992) have involved the analysis of many different traits, such as height, maturity, and even

yield, and have determined a number of chromosomal regions which appear to contain genes with effective factors for these agronomically-important traits. The authors are attempting to develop strategies to provide researchers a means for isolating, cloning, and perhaps even engineering genes involved in these quantitative traits.

Fortunately many of the pathogen resistance traits appear to be as a consequence of a single or a small number of gene products and so are easier to manipulate by genetic engineering, being encoded by a single or a few genes. The source of these genes is often the wild relatives of these cultivated crop plants (Feeney, 1976). The construction of detailed genetic maps for these wild crop species should make the identification of these genes a lot easier.

An interesting development involving the expression of these resistance proteins in transgenic plants is the development of wound inducible promoter systems. Eventually the aim is to produce insecticidal and/or antifungal proteins in response to wounding. The advantages of this inducible response would be that anti pest proteins would only be produced when required, thus saving plant metabolic energy, restricting the production of such proteins to specific plant tissues and possibly increasing consumer acceptability. Additionally, because pathogens would not be continually exposed to the anti-pathogen proteins this would also minimise the chances of insects developing resistance, which is an important consideration when engineering transgenics plants. Xu *et al.* (1992) have used a wound inducible promoter of the potato proteinase inhibitor II gene (*Pin II*) for use in rice transformation. In transgenic rice plants a *pinII-Gus* fusion construct showed a weak wound response. However inclusion of the first intron of the rice actin 1 gene (*Act 1* 5'-intron) in the 5'-transcribed region of the *PinII-Gus* fusion resulted in a high level systemic wound response. The *pinII* fusion constructs have also been found to be expressed in transformed maize, barley and sorghum tissue. Recently a series of optimised *PinII* -based expression vectors for use in cereal transformation have been used to express natural insecticidal proteins in transgenic rice plants.

In conclusion, by exploiting the inherent resistance shown by some plants against insect herbivores and microbial pathogens, the transfer of resistance genes across the barriers of conventional plant breeding, into agronomically important crop plants is a major goal of crop improvement. A considerable amount of research is now channelled into investigation of these resistance traits and identifying the genes which encode for them.

1.3 Plant Defence mechanisms

Plants have evolved several mechanisms to protect themselves against pests and pathogens, including physical barriers, secondary metabolites and antimetabolic proteins.

1.3.1 Plant physical defences

Thick cuticles, specialised trichomes and thick seed integuments are some of the plants physical barriers to pathogen attack. Features such as these are often encoded by complex genetic systems. Although they have been exploited and transferred to modern crops by traditional plant breeding methods (Simmons, 1981), they are not amenable for gene transfer using genetic engineering within the present confines of technology. Several enzymes involved in cell wall synthesis as well as glycine rich proteins and structural proteins such as extensins are believed to be involved in plant defense. They are believed to be provide a physical barrier against invading fungal hyphae and insects (Bowles, 1990).

Pappachan *et al.* (1992) describe one such mechanism which involves deposition and crosslinking of phenolics at the cell surface and further deposition of aliphatic polyester domains to complete suberization in tomato. A highly anionic peroxidase was found to be involved in the deposition of phenolics on the wall. Subsequently, two highly similar genes in tomato were found to encode these anionic peroxidases. Expression of this gene was found to be associated with resistance of a tomato line to *Verticillium albo-atrum*. Transgenic tobacco plants containing the peroxidase promoter fused to GUS showed GUS expression only when induced by wounding or by fungal attack. Transgenic tobacco plants constitutively expressing the tomato anionic peroxidase showed resistance to fungal attack.

1.3.2 Plant secondary metabolite defence

Several studies have been carried out indicating the presence of toxic or antifeedant secondary metabolites in seeds of several plants, which are thought to be involved in their resistance to pathogen attack (Bell, 1981, 1976; Janzen, 1977). Plant secondary metabolites involved in insect resistance are divided into three groups. Firstly, those which are derived from acetate and mevelonate, namely the acetogenins and isoprenoids. These are involved in insect resistance through olfactory feeding deterrants and growth inhibition traits. Secondly are metabolites derived from shikimic acid, and amino acids such as polyphenols and alkaloids such as nicotine, strychnine and atropine. These are highly toxic to several insects and microrganisms (Gatehouse *et al.*, 1991). Lastly are the flavonoids which include compounds such as rotenoids, flavenoids and tannins. The flavenoids are effective feeding deterrents often being very astringent tasting whereas tannins and rotenoids are very toxic when ingested by insects (Birch *et al.*, 1985; Feeny, 1970, 1976).

Many of these proteins and their reactions are essential to processes in normal growth and development of the plant as well as being important in plant defence and accordingly their regulation is complex. This fact in combination with their toxicity to mammals renders it

unlikely that they will be useful for engineering plant resistance by the present technologies available.

1.3.3 Proteins involved in plant defense

Invasion of a plant by its pathogens triggers an altered pattern of protein synthesis in which enzymes are produced, many of which are deliterious to these fungal and insect invaders. These proteins or pathogenesis proteins as they have been named include fungal cell wall degrading enzymes such as hydrolases, β-glucanses, lectins and chitinases (Shapira *et al.*, 1989). It is widely believed that these proteins act directly on the invading pathogens and have subsequently been called pathogenesis related proteins (PR proteins). Many of these proteins are encoded by small gene families and their patterns of expression are well understood. These proteins and subsequently the genes encoding for them have become the subject of study for molecular biologists, protein and genetic engineers with a view to enhancing the natural defense system, and also increasing the productivity and nutritional value of plant foods (Croy and Gatehouse, 1985).

1.3.3.1 Lectins

The existance of lectins in plant has been known for more than 100 years since the discovery by Stillmark of a factor from castor bean capable of agglutinating erythrocytes. Since then they have been characterised as carbohydrate-binding proteins that bind glycans of glycoproteins, glycolipids, or polysaccharides with high affinity (Goldstein *et al.*, 1980; Goldstein and Poretz, 1986). Because of their binding specificity, they have the capability to serve as recognition molecules within the cell, between cells, or between organisms. In plants they are ubiquitous, and are assumed to play fundamental biological roles.

Of the many lectins that have been characterised, most are secretory proteins which subsequently accumulate either in the vacuoles or in the cell wall and intercellular spaces. They have also been isolated from vegative organs such as roots, leaves, rhizomes and stems, some of which are vacuolar in origin whilst others are extracellular. Many biological roles have been proposed for plant lectins (Etzler, 1985). The discovery of different lectins in different tissues of the same plant (Van Damme and Peumans, 1990) may indicate that during evolution lectins have become adapted for different functions in different tissues and that no clear role can be assigned. Chrispeels and Raikhel (1991) believe that the most likely function for the vacuolar lectins is in plant defense although they emphasise that extracellular root lectins may be involved in the recognition of bacteria for the purpose of establishing symbiosis, as first proposed by Bolhool and Schmidt (1974).

There are a number of ways in which vacuolar lectins can interact with molecules within and outside the cell and thus with the pathogen. Firstly when dry seeds imbibe water, vacuolar proteins including lectins are released into the imbibtion water (Fountain *et al.*, 1977) and into the area surrounding the germinating seed, where they can interact with potential pathogens. Secondly when seeds and other lectin containing tissues are eaten by predators, lectins will be released from the disrupted cellular structures of the plant tissue. These lectins may come in contact with the glycoproteins that line the intestinal tracts of the predators, possibly inhibiting absorption of nutrients. Thirdly, when fungal hyphae grow into plant tissues, they may disrupt cellular compartmentation, causing the release of vacuolar lectins that may inhibit further hyphae growth.

There are several reports of lectins restricting fungal growth. Mirelmar *et al.* (1978) reported the inhibition by wheat lectin of growth of the fungal pathogens *Fusarium* and *Trichoderma*.

The lectin arcelin was discovered when Osborn *et al* . (1988) investigated the properties of a protein which is abundant in certain Mexican accesions of the common bean that are resistant to the two bean weevils, *Zabrotes subfasciatus* and *Acanthoscelides obtectus* which are predators of domesticated cultivars. These resistant strains all contain the protein arcelin which is absent from susceptible varieties, indicating that arcelin may be involved in the plants insect resistance strategy.

In a similiar study, Edwards *et al.* (1991) report that a lectin from pea (*Pisum sativum* L.) which belongs to a group of structurally related lectins including the lentil lectin, favin (from *Vicia faba*) and concanavalin A (from *Canavalia enisformis*) has anti-insect properties. This lectin has been shown to be toxic to bruchid beetles when added to an artificial diet, with an LD_{50} of approximately 1.5% of total dietary protein. Pea lectin is known to have extremely low mammalian toxicity, making it an interesting candidate for transgenic expression and potential agronomic improvement.

The gene for this pea lectin has been cloned and partially characterised (Gatehouse *et al.*, 1987). This gene under the control of the CaMV 35S promoter is expressed when transferred to tobacco. The transgenic plants were shown to contain active lectin protein in their leaves at a level of expression suitable to confer resistance to the lepidopteran pest *Heliothis virescens* (tobacco budworm).

The common bean contains a protein called phytohemagglutinin (PHA), an abundant lectin that has been characterised (Goldstein and Hayes, 1978). It was initially believed to be toxic to insects and specifically to bruchid beetles (Janzen *et al.*, 1976). However Murdock *et al.*

(1990) confirmed that this toxicity was specific to an α-amylase inhibitor which was copurified with the PHA preparation.

1.3.3.2 Chitinases

Chitin, a linear β-1,4-linked polymer of N-acetylglucosamine, is a common constituent of fungal cell walls and of the exoskeleton of arthropods. All organisms that contain chitin also contain chitinases (poly-β-1, 4-(2-acedamido-2-deoxy)-D-glucoside glycanohydrolases, (EC 3. 2. 1. 14), which are presumably required for morphogenesis of cell walls and exoskeletons (Gooday, 1977). Other organisms that do not contain chitin may produce chitinases to degrade the polymer for food, for example soil bacteria that secrete chitinases in response to chitin in their environment (Oranussi and Trinci, 1985). Plants also contain chitinases, often after enzyme synthesis has been induced by microbial infection or other injuries (Pegg and Young, 1982; Boller *et al.*, 1983). Since plants do not contain chitin in their cell walls, it has been suggested that they produce chitinase to protect themselves from chitin-containing parasites such as fungi and insects (Abeles *et al.*, 1970; Bell, 1981; Boller, 1988; Boller *et al.*, 1979).

Initial work on chitinases was involved with those of the bacterial origin, Mitchell and Alexander (1961) demonstrated that fungal cell wall lytic bacteria added to soil, controlled *Fusarium* spp. and *Pythium* sp. by destroying the fungal mycelium, whilst Mitchell and Hurwitz (1965) protected tomato plants against damping-off caused by *Pythium aphanidermatum* with lytic *Arthrobacter*. Campbell and Ephgrave (1983) showed that the biological control of *G. graminis* by a *Bacillus sp.* in the soil was due to hyphal lysis. Subsequent to these investigations it was determined that chitinases were the active agents responsible for the resistance.

Following analysis of 100 microorganisms, the enteric soil bacterium *Serratia marcescens* was found to be the most efficient bacterial producer of chitinase (Monreal and Reese, 1969). Subsequently it has been investigated for both its biocontrol properties and as a source of chitinase genes for gene transfer. *S. marcescens* was found to be an effective biocontrol agent against *Sclerotium rolfsii* and *Rhizoctonia solani* under greenhouse conditions (Ordentlich *et al.*, 1987). Additionally, investigations using chitinase preparations isolated from bacteria concluded that the disease control was a direct result of the lytic action of chitinase on fungal hyphae (Ordenlich *et al.*, 1988; Shapira *et al.*, 1989).

Sundheim *et al.* (1988) transferred a plasmid containing a chitinase gene from *S. marcescens* into *P. fluorescens* and, the resulting strain inhibited growth of *Fusarium oxysporum* f. sp. *redlens* and reduced disease of radish which is caused by this pathogen. Similiarly, a *S.*

marcescens chitinase gene transferred to *E.coli* resulted in a strain that was inhibitory to *F.oxysporum* f.sp. *pisi* (Jones *et al.*, 1986).

The idea that plants possess proteins with antibiotic activity, was first demonstrated by Mirelmar *et al.* (1978) who found that a wheat germ chitinase which was contaminating a lectin preparation was responsible for growth inhibition of the fungus *T. viride.* Since then, interest in chitinases and their genes has increased with the aim of elucidating their role in plant defence. Antifungal chitinases may be widely distributed throughout the plant kingdom, either in the stems and leaves following induction by ethylene or pathogen attack (Boller *et al.*, 1983; Boller and Vogeli, 1984; Boller and Metraux, 1988) or stored in the seeds as means of increasing the seed resistance to fungi and insects in the soil (Powning and Irzykiewicz, 1965). These chitinases, by acting directly on growing hyphal tips or in concert with other hydrolyic enzymes (Schlumbaum *et al.*, 1986), may help limit and define the fungal and insect species that can succesfully parasitize plants. The activity of chitinases against fungal pathogens has also been demonstrated by fungal inhibition assays *in vitro.* As yet no reports have been published involving insect bioassays, but it is possible that chitinases could act on the insect cytoskeleton and/or the gut peritrophic membrane.

However, Verburg and Huynh (1990) suggests that the role of chitinase in the plant disease mechanism is to release elicitors of defense related lignification from fungal cell walls. De Jong (1992) concluded that a glycosylated acidic chitinase of *Mr* 32000Da, also elicited, was involved in somatic embryo development in carrot cell cultures. It is possible that an unknown chitinase substrate exists in plants and that these enzymes have an as yet unrecognized role in plant growth and development (Samac and Shah, 1991).

Plant chitinases are usually coded for by small multigene families. Six chitinase isoforms have been described in potato (Kombrink *et al.*, 1988), four in maize (Nasser *et al.*, 1988), four in tobacco (Legrand *et al.*, 1987; Payne *et al.*, 1990) and four in bean (Broglie *et al.*, 1986). Based on the amino acid sequences of these gene products there are at least three classes of plant chitinases (Shinshi *et al.*, 1990). The class I chitinases are basic proteins that contain a highly conserved cysteine and glycine rich N-terminal domain with putative chitin binding properties. Expression of the class I chitinases from bean and tobacco is induced by ethylene (Broglie *et al.*, 1986) and the mature enzyme appears to be located in the plant cell vacuole (Boller and Vogeli, 1984). The class II enzymes have a high degree of sequence similiarity to class I enzymes but lack the chitin binding domain and are acidic proteins (Shinshi *et al.*, 1990). Class II chitinases from tobacco are found in intercellular washing fluids (Parent, 1985) and therefore are probably associated with the cell wall or excreted in the apoplasm. Class III chitinases lack sequence similiarity to class I or II enzymes and may

be basic or acidic proteins. Enzymes in class III include the acidic extracellular cucumber chitinase (Metraux *et al.*, 1988, 1989) and the *Hevea* latex chitinase (Rozeboom *et al.*, 1990).

The specific roles that these different classes of chitinases play in plant defense against pathogens has been investigated by Carr and Klessig (1989) and Boller (1988). They proposed that the class II and III chitinases, which are extracellular forms, are part of an early, induced response. They may act directly by blocking the growth of hyphae invading the intracellular spaces and possibly indirectly as well by releasing fungal elicitors which than induce the host hypersensitive reaction. The class I chitinases, on the other hand, are localised inside cells. They accumulate at high concentrations in the leaf epidermis of uninfected plants and are induced by pathogen infection (Meins and Ahl, 1989; Vogeli-Lange *et al.*, 1988). These chitinases are thought to act following wounding as part of the constitutive defense reaction and late in the infection process when cell breakage releases the enzyme into the extracellular compartment.

Work carried out by Samac and Shah (1991) investigated the expression of the *Arabidopsis* acidic chitinase promoter in response to fungal pathogens. A chimeric gene composed of 1.129 Kb of 5' upstream sequence from the acidic chitinase gene was fused to β-glucuronidase (GUS) coding region and used to transform *Arapidopsis* and tomato plants. Promoter analysis was monitored by histochemical and quantitative assays of GUS activity. In healthy transgenic plants the acidic chitinase promoter activity was restricted to roots, leaf vascular tissue, hydathodes, guard cells, and anthers, whereas GUS expression was induced in mesophyll cells surrounding lesions caused by *Rhizoctonia solani* infection of transgenic *Arabidopsis*. In transgenic tomato plants, GUS expression was induced around necrotic lesions caused by *Alternaria solani* and *Phytophera infestans.*; thus showing that the expression of some chitinase genes is induced by pathogen invasion.

Similiarly, the temporal and spatial expression of the bean chitinase promoter has been investigated in response to fungal attack (Roby *et al.*, 1990). Analysis of transgenic tobacco plants containing a chimeric gene composed of a 1.7 Kb fragment carrying the chitinase 5B gene fused to the coding region of the gus A gene indicated that the chitinase promoter is activated during attack by the fungal pathogens *Botrytis cinerea, Rhizoctonia solani* and *Sclerotium rolfsii*. These results strengthen the argument that chitinases are involved in plant defence.

Recently the potential for exploiting the genes encoding these chitinases for use in crop protection has been realised. Transfer of chitinase genes into important crop plants and their subsequent overexpression could increase the plants resistance to fungal and insect

predation. An important point to consider is that in many cases, chitinases and β-1, 3-glucanses are coordinately induced by ethylene or by pathogen attack in plants. It has been suggested that chitinase is only an effective fungicide *in vitro* when applied in combination with β-1, 3-glucanse (Mauch *et al.*, 1988a, 1988b; Schlumbaum *et al.*, 1986). It is worth bearing in mind that in the construction of pathogen resistant plants it may be necessary to cotransfer these two genes.

Initial work has concentrated on bacterial chitinases as the source of transferable genes. The *chiA* gene of *Serratia marcescens* (Jones *et al.*, 1986) codes for the secreted protein, bacterial chitinase (*ChiA*). Lund *et al.* (1989) investigated the modifications and the cellular location of *ChiA* when it is expressed in transgenic tobacco plants. The results show that bacterial chitinases can be expressed succesfully in plant cells. Unfortunately, no trials were carried out to determine whether the chitinase confers any fungal resistance in tobacco following pathogen invasion. Additionally, *ChiA* from *S. marcescens* was succesfully expressed in tobacco leaves under the control of both the ribulose bisphosphate carboxylase small subunit (*rbs*S) promoter (Jones *et al.*, 1988) and the 35S promoter from cauliflower mosaic virus (Lund*et al.*, 1989). Jach *et al.* (1992) succesfully transferred and expressed the *ChiA* gene from *S. marcescens* in transgenic tobacco plants. Trials with the fungal pathogen *Rhizctonia solani*, which causes root and stem infections confirmed that transgenic plants expressing *ChiA* are more resistant than the control tobacco plants to invasion by this fungus. Additionally, following analysis of the offspring of selfed *ChiA* transgenic plants it was determined that the fungal resistance was stabely inherited .

In contradiction to the above work, controversial work carried out by Roberts and Selitrennikoff (1988) suggests a preference for plant derived chitinase genes for fungal inhibition. They compared the hydrolytic mechanisms of bacterial and plant chitinases and concluded that the antifungal activity of bacterial chitinases differs from plant chitinases in their mechanism of action. The consequence of which is important for engineering resistant plants.

Two antifungal proteins were isolated from barley grain (Roberts, 1986). The purified chitinases from barley, along with proteins from maize, wheat and *S . grieus*, and the cloned chitinase gene products from *S. marcescens* and *P. stutzeri*, were tested for antifungal activity by their ability to inhibit hyphal extension. The authors concluded that bacterial chitinases could not inhibit fungal growth, although they were able to hydrolyse chitin (at least the purified, partially degraded chitins used as substrates in the assay). The barley chitinase inhibited the growth of *Trichoderme reesi*, *Alternaria alternaria*, *Phycomyces*

blakesleeanus and *Neurospora crassa*. This contradicts work discussed previously, carried out by several other researchers.

Bacterial chitinases from *S griseus* and *S. marcescens* have been shown to degrade chitin by an exohydrolyic mechanism (Molano *et al.*, 1977). In contrast, chitinases from wheat germ (Molano *et al..*, 1979) and bean leaf (Boller *et al.*, 1983) degrade chitin by an endohydrolytic mechanism. Roberts and Selitrennikoff (1988) suggested that one explanation for the fact that antifungal activity is limited to the grain chitinases is that these enzymes function as endochitinases and can cleave any portion of a chitin polymer with which it can come in contact. The bacterial chitinases being exochitinases, are restricted to locating non reducing termini of chitin as substrates, which may be difficult in the intact fungal cell wall. Inaccesibilty of termini may also play a role in the inability of exochitinases, but not endochitinases, to hydrolyse bacterial cell walls, although they suggest that this difference in specificity could be due to other factors. The inability of exochitinases to inhibit fungal growth suggests that any future experiments designed to introduce chitinase genes into plants to improve their resistance to fungal attack should use chitinase genes from other plants, rather than from bacteria.

Neuhaus *et al.* (1991) examined the effect of high level expression of a tobacco chitinase gene in *Nicotiana sylvestris* by analysing the susceptibility of transgenic plants to *Cercospora nicotianae* infection. A gene for class I (basic) tobacco chitinase regulated by cauliflower mosaic virus 35S-RNA expression signals was transferred into *Nicotiana sylvestris*. The chitinase was correctly processed to produce enzymatically active chitinase targeted to the intracellular compartment of leaves. Most transformants accumulated extremely high levels of chitinase; up to 120 fold that of non-transformed plants in comparable tissues. Unfortunately, high levels of chitinase in transformants did not substantially increase resistance to the chitin containing fungus *Cercospora nicotiana*, which causes frog eye disesase. Therefore, class I chitinase does not appear to be the limiting factor in the defense reaction to this pathogen. One may speculate whether cotransfer of this chitinase gene with a gene encoding for a β-1, 3-glucanase would decrease susceptibilty to attack by this fungi. Alternatively Jach *et al.* (1992) speculate that the problem of over expressing endogeneous chitinases is that the fungus may have become adapted to the defence mechanisms of the plant and can resist the overexpression of the chitinase gene. The authors suggest that chitinases from non-related plants should be used with the assumption that the invading fungus should not adapt to the effect of these proteins.

The isolation of genes expressing plant endochitinases continues and it remains to be seen whether overexpression of genes encoding for other chitinases will increase the resistance of plants to pathogen attack.

1.3.3.3 Ribosome inactivating proteins

Ribosome inactivating proteins (RIPs) are a commonly distributed family of highly toxic plant proteins that catalytically inactivate eukaryotic ribosomes thus blocking translation (Stirpe and Barbieri, 1986). RIP activity has been found in the seed, root, leaf, or sap of more than 50 plant species, and have been tested extensively for their effects on protozoa, insects and fungi. Gatehouse *et al.* (1990a) demonstrated that both type II (two subunit) and Type I (single subunit) RIPs were toxic towards members of the Coleoptera (for example, the bruchid beetle) and Orthoptera (for example, locust) but not the Lepidopteran pests tested.

Logemann *et al.* (1992a, 1992b) showed that expression of a barley RIP under the control of the potato *wunI* gene resulted in protection from the soil borne pathogen, *R. Solani* in transgenic tobacco plants. Interestingly, *in vitro* studies with *Trichoderma reesi* and *Fusarium sporotrichloides* demonstrated that a combination of barley RIP and chitinase inhibits fungal growth more efficiently than either of the enzymes alone. The authors suggest that the fungal cell wall degrading action of the chitinases allows uptake of RIPS to be increased resulting in the increase in fungal inhibition.

1.3.3.4 Thionins

A family of small (M*r* approx. 5000Da) polypeptides known as thionins occur in both monocotyledonous and several dicotyledonous plants (Bohlman *et al.*, 1988). Most thionins studied so far have been shown to be highly toxic to various bacteria, fungi and small animals. Bohlman *et al.* (1988) described a novel class of thionins with antifungal activity present in the cell wall of barley leaves and in the protein bodies of the seed endosperm. They investigated the toxicity of leaf specific thionins of barley towards the two fungi *Thievaliopsis paradoxa*, a pathogen of sugar cane and *Drechslera teres*, a pathogen of barley. The leaf specific thionins extracted from barley were effective in suppressing the growth of the fungus. This along with the fact that their synthesis can be triggered by pathogens and under certain conditions by other stress suggests that these polypeptides may be important as an inducible defence factor involved in the general resistance of barley.

1.3.3.5 Zeamatins

Roberts and Selitrennikoff (1990) purified a 22 kDa antifungal protein from *Zea mays* seeds, named zeamatin. It was purified to homogeneity and assays showed that it had the unusual property of acting synergistically with nikkomycin to inhibit growth of *Candida albicans*. Alone it inhibited growth in suspension culture of *C. albicans*, *Neurospora crassa* and *Trichoderma reesei* . The authors suggest that zeamatin is not an enzyme, but a protein that exerts its antifungal effects by damaging fungal membranes. The authors also purified from sorghum an antifungal protein that is similiar, but not identical to zeamatin and suggest that this may be a new family of antifungal proteins which may have potential importance for engineering fungal resistance in plants.

1.3.3.6 Lipoxygenases

Lipoxygenase(s) catalyse the formation of fatty acid hydroperoxides which are metabolised to a range of compounds with different physiological effects and organoleptic properties. Although their biological role is unclear lipoxygenases are implicated in the plants response to pathogen and pest attack, wounding and stress. Shukle and Murdock (1983) have reported an antimetabolic effect of lipoxygenase from soybean on *Manduca sexta* (a non pest species) by artificial diet bioassay.

1.3.3.7 Salicylic acid response 8.2 protein family

SAR 8.2 is a previously uncharacterised PR protein gene family which is comprised of at least 5 expressed members. The family is inducible by TMV inoculation, as well as by salicylic acid treatment, in *Nicotiana tabacum* cv. Xanthi nc. Computer analysis predicts that the SAR 8.2 proteins are small (7.5 to 9.6 kd), and highly basic (pI 10.0-10.8). The C-terminal domain of the 8.2 protein has homology to metallothioneins and other metal-binding proteins. Glascock *et al.* (1992) recently reported evidence for protection against *Phytophthora parasitica pv. nicotiana* during analysis of plants expressing constitutively the SAR 8.2 cDNA.

1.4 Proteinaceous inhibitors of enzymes

Within plants are a range of proteins which act as natural inhibitors for a variety of enzymes such as: trypsin, chymotrypsin, subtilisin, papain, bromelain, α-amylases, transferases (Garcia-Olmedo *et al.*, 1987; Richardson, 1991; Liener and Kakade, 1980; Weder, 1986; Buonocore and Silano, 1986; Rackis *et al.*, 1986). These inhibitors have been found in a variety of sources including the fruits, tubers and seeds of many plant species. Apart from their general role in seed metabolism by regulating the activity of their target proteins it has been suggested that these proteins may have several other roles in plants. The implication is

that these inhibitors are involved in a general defence mechanism against tissue damage, and invasion and consumption by predators (Ryan 1973, 1981, 1989; Richardson 1991; Richardson *et al.*, 1987a; Wilson 1980; Garcia-Olmedo *et al.*, 1987).

For example, these proteins by inhibiting extracellular enzymes, may retard or prevent the invasion of plant tissues by saprophytic and parasitic microorganisms such as fungi and bacteria, and insect invasion. Another theory is that these protein inhibitors may make seeds containing them less pallatable even lethal to insect predators which can attack them over wintering, periods of dormancy or even storage (Birk and Applebaum, 1960; Birk, 1961).

This idea of protein inhibitors involved in plant defense seems to have recieved some support from the results obtained from feeding trials with insects (Gatehouse *et al.*, 1986, 1990a; Ishimoto and Kitamura, 1988). *In vitro* many of these inhibitors are effective against some of the digestive enzymes in the guts of insects which are common plant invaders. The effect is to interfere with normal development of the larvae and inflict high mortality rates (Gatehouse and Boulter, 1983). However there is no evidence that indicates that these deleterious effects are related to the *in vivo* action of the inhibitors against the target enzymes from these insects (Gatehouse *et al.*, 1990a, 1990b). In no case has the complex of the enzyme inhibitor fed to the insect and its target enzyme ever been isolated following ingestion.

1.5 The cow pea trypsin inhibitor

An example of plant proteinaceous inhibitors conferring resistance against pathogens is illustrated by the cow pea trypsin inhibitor, a proteinase inhibitor present in the cowpea seed (*Vigna unguiculata*), a grain legume. The purified trypsin inhibitor was found to be an effective insecticide against a wide range of economically important field and storage insect pests all of which cause crop losses of major economic importance (Gatehouse *et al.*, 1990b).

Boulter *et al.* (1990), transformed tobacco (*Nicotiana Tabacum*) with the cow pea trypsin inhibitor gene (Hilder *et al.*, 1987), which was under the control of a CaMV-35S promoter (Guilley *et al.*, 1982), by *Agrobacterium tumefaciens* mediated gene transfer. Insect bioassays on the plants expressing this gene showed a negative effect on the growth, and an increase in the mortality rates of *Heliothesis virescens* (tobacco budworm). This was the first successful example of the conferring of insect resistance to a transgenic plant using a genes of plant origin.

1.6 The α–amylase inhibitors

Studies at the beginning of the 1930's (Chrazaszcz and Janicki, 1933), confirmed later by Kneen and Standstedt (1943, 1946), demonstrated the presence of plant protein inhibitors of α–amylase (1, 4-α-D-glucan glucanohydrolase, E.C. 3.2.1.1) in extracts of various cereals. Their target enzymes are α–amylases, which are enzymes that catalyse an α(1-4) endoglycolytic cleavage of amylose and amylopectin. Shortly afterwards Kunitz (1945) isolated and purified a heat labile protein from soybean which inhibited trypsin. Since then interest in these plant protein inhibitors has increased dramatically bearing in mind their therapeutic, biochemical, nutritional and agricultural properties (Ryan, 1973, 1979, 1981, 1984; Richardson, 1977, 1981, 1991; Liener and Kakade, 1980; Laskowski and Kato, 1980; Gatehouse *et al.*, 1984; Silano *et al.*, 1973; Buonocore and Silano, 1986; Rackis *et al.*, 1986; Weder, 1986; Garcia-Olmedo et al., 1987). Current opinion is that these proteins may have a role as defensive agents defending the plant against the attack of insects and microbial pests (Green and Ryan 1972, Ryan 1973, Brown *et al* 1986) and therefore may have potential for crop improvement by genetic engineering.

Due to molecular similarities and other common features, these inhibitors are grouped into 10 different families, distributed across many species and tissue types including plants, animals, microorganisms and in the fruits, and tubers of many plants. The majority of α-amylase inhibitors from plants have been placed in 4 well-defined families, including the cereal superfamily (Shrewry *et al.*, 1984). To date α-amylase inhibitors have been purified from several cereals including wheat (*Triticum*), barley (*Hordeum*), ryes (*Secale*), corn (*Zea mays*), oats (*Avena sativa*), ragi (*Eleusine coracan*), rice (*Oryza sativa*), *Coix* (*Coix lachryma-jobi*), sorghum (*Sorghum bicolor*) and also from legumes such as bean (*Phaeseolus sp.*), chick pea (*Cicer arietinum* L.) and peanut (*Arachos hypogea*). Studies on the structure of numerous enzymes inhibitors found in other sources show that this family is distributed beyond the plant kingdom (Odani *et al.*, 1983; Richardson, 1991).

1.6.1 Distribution and inhibitory activities of α–amylase inhibitors

The Kneen and Sandstedt (1943, 1946) inhibitor was located in the endosperm, this was confirmed by Buonocore *et al.* (1977) and Buonocore (1977) who concluded that α-amylase inhibitors were endosperm specific. Synthesis of this protein family seems to precede that of the bulk of the reserve proteins and starch. The production of the wheat inhibitor with kernel development was followed qualitatively by Sandstedt and Beckford (1946) and quantitatively by Pace *et al.* (1978). Synthesis of the inhibitor starts about 8 days after fertilisation and rapidly increases with maturation up to a maximum reached at the full

maturity. The inhibitor content rapidly decreases after germination; no detectable amount was observed in roots and coleoptiles from germinated seeds (Pace *et al.*, 1978). Following these observations he suggested that these inhibitors do not play a specific role during germination.

Moreno and Chrispeels (1989) isolated a glycoprotein, α-amylase inhibitor from *P. vulgaris* which inhibits both mammalian and insect α-amylases. The α-amylase inhibitor is present in the embryonic axis and cotyledons, but not in other organs (i.e. seed coats and pods, leaves, stems, and roots). The protein is synthesised during the same time period as phaseolin and phytohemagglutinin are synthesised, which is 17 dpa, and accumulates in the protein storage vacuoles where the amount increased until it reaches a plateau approximatelyt 27 dpa. The inhibitor content remained the same or decreased slightly (on a dry weight basis) during the drying of the seed. This α-amylase inhibitor is a vacuolar protein, which accumulates in the vacuole following synthesis as a preprotein on the rough endoplasmic reticulum, transport to the vacuoles is mediated by the Golgi.

In a study of *in vivo* and *in vitro* synthesis of α-amylase inhibitors in barley Paz-Arez *et al.* (1983) concluded that synthesis took place between 10 and 30 daa, with a peak between 15 and 20 days after anthesis. These proteins were synthesised by membrane bound polysomes as precursors of higher apparent Mr (13,000-21,000) than the mature proteins (12,000-16,000). The largest *in vitro* product of 21,000 was found to be a precursor of the barley α-amylase inhibitor subunit CMd.

1.6.2 Inhibitor specificity

Garcia-Olmedo *et al.* (1987) describe these inhibitors as highly specific substrates for their target enzymes, which undergo a limited and extremely slow proteolysis, so that the system behaves as if the free enzyme and the inhibitor were in simple equilibrium with the enzyme/inhibitor complex. On the surface of the inhibitor there is at least one reactive bond which interacts with the active site of the enzyme. The majority have the reactive site peptide bond contained within at least one disulphide loop, which ensures that during conversion of the original to the modified inhibitor the 2 peptide chains do not dissociate. The nature of the amino acid residue at the carboxyl site generally determines the proteinase inhibited (Laskowski and Kato, 1980). More than one reactive site is sometimes present in a single peptide chain, in which case more than one enzyme molecule of the same or different specificity can be simultaneously inhibited by a single molecule. Structural analysis of these "multi-headed" inhibitors revealed the presence of 2-7 repeating sequences, each of which contain reactive binding site for a proteinase (Odani *et al.*, 1986). A single species of plant or

animal usually contains a set of many inhibitor variants, which in spite of their closely resembling structures, show a wide variety of inhibitor spectra resulting from minor changes of amino acid sequence around the reactive site. Garcia-Olmedo *et al.* (1987) suggest that the divergent evolution of proteinase inhibitors occured from a limited number of ancestors by fused or separate gene multiplication and mutation events.

The diversity of target amylases which can be inhibited is illustrated by studies concerning the inhibitor of Kneen and Sandstedt (1943, 1946) which inhibited several amylases from *Streptomyces* and some insect amylases including those from *Prodenia litura* and *Tribolium castaneum* . It was not active against barley and sorghum α-amylases and barley β-amylase.

The fact that many of these inhibitors do not appear to inhibit their own plant amylases *in vitro* despite being inhibitory against other amylases from varied sources has led to the idea that these proteins are involved in plant defence, protecting the plant against damage from insects, fungi and microorganisms.

The α-amylase inhibitors are found in a number of plant species but information is most complete for wheat and barley as described below.

1.7 The Wheat α-amylase inhibitor

Since the initial characterisation of the wheat flour α-amylase inhibitor by Kneen and Sandstedt (1943, 1946) several others have been characterised from seeds of numerous *Triticum* and related *Aegilops* species. The wheat inhibitors have been classified into three classes, namely the monomeric, dimeric and tetrameric inhibitors. All of these proteins showed different inhibitory profiles when tested with several α-amylases. For example the dimeric inhibitor, inhibits mainly α-amylase from human saliva, whereas all three inhibitors were active against insect α-amylases from *Tenebrio molitar* (Buonocore *et al.*, 1977).

Isolation of these inhibitors and their subsequent characterisation was undertaken by several workers (Shainkin and Birk, 1970; Saunders and Lang, 1973; Silano *et al.*, 1973; Petrucci *et al.*, 1976, 1978; O'Donnel and McGeeney, 1976; O'Connor and McGeeney, 1981; Maeda *et al.*, 1982). The first two types of inhibitors characterised were assigned the 0.28 and 0.19 inhibitor families. The 0.28 family included the monomeric variants of about M*r* 12,000Da. When assayed, these were more active against insect α-amylase inhibitors than against human salivary or pancreatic amylases. The 0.19 family includes the dimeric inhibitors which have approximately M*r* 24,000Da. These inhibitors are highly active against insect amylases. Silano *et al.* (1973) concluded that these dimeric proteins could be dissociated into

identical 12,000Da subunits. Both subunits contain one molecule of carbohydrate per subunits which were originally thought to be important with respect to their inhibitory functions (Silano *et al.*, 1977) but this is now being disputed. O'Connor and McGeeney (1981) investigated the wheat tetrameric inhibitor which has a *Mr* 63,000Da. Following dissociation of this protein, subunits of Mr 14,000 and 15,000Da were detected. No carbohydrate moities have been found. This inhibitor is active against insect and human but not bacterial amylases.

Deponte *et al.* (1976) confirmed the existance of these three families when 3 albumin fractions were discovered with apparent molecular weights 60000, 24000 and 12000Da following gel filtration chromatography of wheat protein extracts, precipitated with ammonium sulphate.

The stochiometries of the inhibitor/enzyme complex were investigated and found to be 2:1 for monomeric and 1:1 for the dimeric inhibitors.

An extensive investigation of inhibitor properties of the 3 size classes of inhibitors versus α-amylase from 18 insects, 23 marine, and 17 avian and mammalian species were carried out by Silano *et al.* (1975). The monomeric were most effective against insect enzymes particularly against marine, avian and mammalian amylases, there was no clear inhibitory pattern for tetrameric inhibitors. Resistant amylases were largely distributed in cereal, avian and mammalian species as well as in insect species that do not usually wheat grain or wheat flour products.

Warchalewski (1977a, 1977b) was first to characterise inhibitors of endogenous α-amylases in wheat. These inhibitors were highly active against α-amylases from the same source but less active against amylases from other types of wheat, indicating a highly specific interaction between the *a*-amylase and its natural protein inhibitor.

The first complete amino acid sequence of an α-amylase inhibitor to be determined was that of the monomeric 13kDa from wheat (Kashlan and Richardson, 1981).

1.8 The Barley α-amylase inhibitor

Sanchez-Monge *et al.* (1986) demonstrated the existence in barley of 3 families very similiar to the wheat families with Mr 60000, 24000 and 12000Da, these have been termed the CM proteins. Weselake *et al.* (1983a, 1983b) and Mundy *et al.* (1983) were the first to identify an α-amylase inhibitor in barley. Both investigations resulted in the purification of a bifunctional inhibitor termed BASI, which was found to be inhibitory against the barley malt

α-amylase and microbial subtilisin. Lazaro (1988) isolated a dimeric inhibitor from barley which was found to inhibit insect α-amylases.

1.9 α-amylase inhibitors in microrganisms

A number of microorganisms including the *Actinomycetales* order (Ohyama and Murao, 1977), *Streptomyces* genus (Murai *et al.*, 1985) and *Cladosporium herbarum* (Goto *et al.*, 1983; Ashauer *et al.*, 1984) produce compounds active against α-amylases. Only the inhibitors from *S.tendae* 4158/Tendamisit-HOE 467 have been fully characterised and their three-dimensional structures determined. Their restricted specificity towards mammalian α-amylases suggest a common inhibitory pattern for these molecules (Ashauer *et al.*, 1984).

1.10 Bifunctional protein inhibitors

Interest in plant protein inhibitors was stimulated following the discovery that some have surprising sequence homologies with other proteins and enzymes. For example, a bifunctional inhibitor of trypsin and α-amylase from maize seeds (Richardson *et al.*, 1987) has strong sequence similarity with the sweet potato thaumatin, virus induced pathogenesis-related proteins and plant proteins induced by salt and water stress. Additionally the further discovery that an amylase inhibitor from bean (Moreno and Chrispeels, 1989) has the same structure as lectin (agglutinin)-like proteins tend to confirm the suspicion that many of these inhibitor proteins may have other as yet unsuspected functions.

Bloch and Richardson, (1991) isolated three isoinhibitors from the seeds of *Sorghum bicolor* (L) *Moench* which inhibited locust and cockroach gut α-amylase. The 3 inhibitors have strong sequence similarity with the γ-purothionins recently isolated from the wheat endosperm which in turn are related to various toxic plant thionins (toxic to insect larvae) that modify membrane permeability and inhibit *in vitro* protein synthesis in cell free systems.

Wheat (*Triticum aestivum*) contains an endogenous α-amylase inhibitor, WASI which has also been shown to be a subtilisin inhibitor. The protein consists of a single polypeptide with M*r* 20,500Da and has 31% sequence homology to Kunitz type trypsin inhibitor in soybean.

Additionally Zemke *et al.*. (1991) determined that the wheat proteinase inhibitor pK13 is inhibitory against α-amylases from wheat and several insects as well as inhibiting proteinase K. The fact that inhibitory activity against α-amylases is retained after incubation with proteinase K suggests that it has two different binding sites.

Work carried out by Weselake *et al.* (1983a, 1983b) and Mundy *et al.* (1983) identified a bifunctional amylase and subtilisin inhibitor in barley. This 10,000Da protein (Svensson *et*

al., 1986) was homologous with an α-amylase inhibitor from Indian finger millet (Campos and Richardson, 1984). This was cloned and its primary structure obtained by cDNA (Mundy and Rogers, 1986) and amino acid sequence (Svendsen *et al.*, 1986). The amino acid sequence is 50% homologous with α-amylase inhibitor I-2 from Ragi seeds and has distant homology with two Bowman-Birk protease inhibitors. The protein has strong homology with phospholipid transfer proteins (Bernard and Sommerville, 1989) and was shown to be capable of transferring phophotidyl choline in liposomes to potato mitochondria.

Shivarej and Pattabiraman (1980, 1981) isolated a bifunctional inhibitor from Ragi (*Eleusine coracana*) which is able to inhibit α-amylase and trypsin independently by forming a tertiary complex with the 2 enzymes.

1.11 Role of α-amylase inhibitors *in vivo*

1.11.1 Biological role

Only those few inhibitors that have been shown to affect endogenous amylases are likely to play specific roles in plant metabolism. During cereal seed germination, a number of hydrolytic enzymes including α-amylases and proteases are produced to hydrolyse starch and proteins to provide the energy and building blocks required for seedling growth. The inhibitors may be involved in the control of endogenous α-amylases, thus preventing the premature hydrolysis of reserve material.

Cellular localisation of the inhibitors in seeds indicate that some of these proteins are found along with storage proteins in protein bodies and it has been postulated that these α-amylase inhibitors may have a storage role in the seed. In the case of the wheat inhibitors they are closely associated with starch in the endosperm (Buonocore *et al.*, 1977).

In a similiar study, Simmonds *et al.* (1973) reported that the wheat inhibitors strongly adhere to starch granules in the endosperm and make a significant contribution to endosperm hardness playing the role of cementing the substances between starch granules and storage proteins. The speculation can be made that although inactive towards wheat α-amylase *in vitro*, albumin amylase inhibitors affect, during maturation of the kernel, starch digestion by preventing the access of amylase to starch granules.

In addition, the high content of cysteine and cystine found in many plants inhibitors has led to postulate that they are sulphur stores (Hartl *et al.*, 1986).

1.11.2 Protection from environmental stress

It has been suggested that α-amylase inhibitors may play a role in protecting the plant against enviromental stress (Robertson, 1989). Plants exposed to water stress undergo adaptation to the condition by physiological and biochemical responses; the implication being that abscisic acid is promoting the synthesis of a unique set of proteins, including α-amylase inhibitors, that may have some functional role in protecting the plant from this imposed stress. These α-amylase inhibitors may play a role in protecting the germinating seed and seedling from dehydration or other enviromental stresses.

1.11.3 Defensive role against pests

Many plants respond to pathogenic invasion with an altered pattern of protein synthesis in which enzymes are produced whose apparent function is to inhibit the growth of parasitic invaders. Plant proteinaceous inhibitors are thought to be part of the plants defensive arsenal for protection against these invaders.

Many of these inhibitors function by inhibiting some of the digestive enzymes in the gut of insects which are their common predators (Silano *et al.*, 1975; Gatehouse *et al.*, 1979, 1986, 1990; Baker, 1982, 1987, 1988; Yetter and Saunders, 1979; Blanco-Labra and Iturbe-Chinas, 1981). The inhibition of hydrolytic enzymes from predators may be directly toxic or even lethal to them, or could affect their fitness in such away that different feeding habits may evolve. It has been observed that insects which could feed on a tissue like the cereal endosperm, had a higher concentration of α-amylase inhibitors and, higher gut amylases activity. Presumably this overcomes the potentially detrimental effect of the endosperm inhibitors.

Reese (1983) concluded that the anti-nutritional effects of proteinase inhibitors were due to the reduced digestion of dietary protein produced by a direct inhibition of host proteinases. Feeding trials with purified inhibitors added to artificial diets showed that seed proteinase inhibitors do significantly reduce the growth and development of various insect pests (Gatehouse and Boulter, 1983; Broadway and Dufley, 1986). Baker (1988) purified the 12,000 and 24,000Da wheat α-amylase isoinhibitors which were found to be particularly active against amylases from graniverous insects, such as *Sitophilus, Tenebrio, and Tribolium.* Although *Sitophilus* weevils have extremely active midgut amylases it appears that these α-amylase inhibitors are detrimental to their feeding. Lowered amylase levels in eight insect strains, feeding on wheat, relative to amylase level among weevils feeding on barley, corn, or rice may be due to these abundant inhibitors and formation of inactive enzyme-inhibitor complexes with previously secreted amylase.

The α-amylase inhibitor present in the seeds of kidney and azuki beans may explain the resistance of these seeds to the bruchid weevils *Callosobrochus chinensis* (Ishimoto and Kitamura, 1988). Purified α-amylase inhibitors in artificial diets suppressed the α-amylase activity in the midguts of larvae killing the beetles prior to their second instar.

Similar work was carried out by Applebaum (1965) who showed that the addition of crude protein extract from wheat bran to a synthetic diet adversely affected development and greatly increased the mortality of *T. molitor* larvae. The author attributed such affect to the *in vivo* inhibition of the insect amylase by wheat protein inhibitors. The hypothesis that albumin inhibitors naturally occurring in the wheat might provide a measure of insect resistance is also supported by the results of Silano *et al.* (1977) who found that the most of the insects that attack wheat grain and flour seem to have high amylase activities, whereas insect species that do not normally feed on wheat had relatively low amylase activities and amylases resistant to inhibition by wheat albumins.

1.11.4 Defense against fungi and bacteria

Plant pathogenic fungi and bacteria produce extracellular proteinases that play a role in invasion process. Many seed protein inhibitors are active *in vitro* against such microbial produced proteinases (Richardson, 1991). Peng and Black (1976) showed that proteinase inhibitor levels in tomato plants increased following infection by pathogenic fungi *Phytophera infestans*, but this host response only occurred in those varieties of tomato which were resistant to the fungi in the susceptible varieties.

1.12 α-amylase inhibitor coding genes

Interest in the genes encoding these proteinaceous inhibitors has increased rapidly since the realisation of their potential for gene transfer into crop plants by genetic engineering, to confer pest resistance. To date genes encoding α-amylase inhibitors have been isolated from wheat, barley and bean as described below.

Garcia-Maroto *et al.* (1990) isolated 3 cDNA clones obtaines for CM1, CM3 and CM16, which represent the three types of subunits of the wheat tetrameric inhibitor of insect α-amylases. Southern blot analysis of appropriate aneuploids, using the cloned cDNAs as probes, has revealed the location of genes for subunits of the CM3 and the CM16 type within a few kb of each other in chromosomes 4A, 4B and 4D, and those for the CM1 type of subunit in chromosomes 7A, 7B and 7D. Garcia-Maroto *et al.* (1990) suggests that the three types of subunits of the tetrameric inhibitor were originated by a tandem duplication in

chromosome 4 and by translocation between chromosomes 4 and 7. Insertions (or deletions) modified the gene from the primitive subunit as it was duplicated and translocated.

Rasmussen and Johansson (1992) sequenced a cDNA clone coding for the barley seed protein CMa, which is an inhibitor of an insect α-amylase. The clone was isolated from endosperm tissue 15 and 20 dap, it encoded an amino acid sequence of 119 residues preceded by a signal peptide of 25 amino acids. There is a strong preference for a C and G in the third position of the codon as noted for many genes encoding a hydrolytic enzymes of the barley seeds, probably reflecting a high translation rate. CMa was found to be 60-85% identical with the wheat α-amylase inhibitor, but shows less than 50% identity to trypsin inhibitors of barley and wheat. This barley CMa protein, M*r* 13,000 was shown to inhibit α-amylase from *Tenebrio melitor*, the mealworm beetle. Larvae of this can cause considerable damage during grain storage and it is foreseeable that this gene may be used for crop protection against this pest.

Leah and Mundy (1989) determined the nucleotide sequence of the bifunctional α-amylase/subtilisin inhibitor from barley endosperm. The full length cDNA clone encoding BASI was determined as 807 bp, with an open reading frame coding for a protein 203 amino acids in length. The first 22 amino acids correspond to signal peptide, as is the case with several other plant protease inhibitors which are directed to the rough endoplasmic reticulum. BASI is synthesised on membrane bound polysomes as are other protease inhibitors containing an N-terminal sequence. Northern blot hybridisation indicates that BASI mRNA accumulation is strictly tissue specific and is developmentally regulated. BASI mRNA transcripts were only detected in developing starchy endosperm tissue from 14 days after flowering and in the aleurone tissue of germinating seeds. Due to its strong inhibition of subtilisin, it seems likely that the BASI defends the stored seed reserves against degradation by bacterial proteases and so may be used to engineer bacterial resistant crops.

1.13 The construction of pathogen resistant crops using genes encoding for α-amylase inhibitors

As aforementioned proteinaceous inhibitors and inhibitors of α-amylases may play a protective role against the attack of animal predators, insects, fungi, bacteria and viruses and are therefore considered to be potential protective agents which are susceptible to genetic manipulation by recombinant DNA techniques. The feasibilty of this actually being applied is demonstrated by Altabella and Chrispeels (1990) who transformed tobacco plants with the bean α–amylase inhibitor gene which was expressed to produce an inhibitor of insect α–amylase in their seeds as described below.

Phaseolus vulgaris L. seeds contain an α-amylase inhibitor which is a thermostable glycoprotein, molecular mass 45,000Da (gel filtration) and is composed of subunits of 15-18,000Da (SDS-PAGE). The α-amylase inhibitor is synthesised as a 28,000Da polypeptide which becomes glycosylated to a series of glycoforms (30-35,000Da) which are then proteolytically processed. It forms a 1:1 complex with insect and mammalian α-amylases, but is inactive against plant and bacterial amylases *in vitro*. Moreno and Chrispeels (1989) presented strong evidence that this inhibitor is encoded by an already identified lectin gene, whose product although an inhibitor of α-amylase is referred to as a lectin like protein (LLP).

Altabella and Chrispeels (1990) made a chimeric gene consisting of the coding sequence of the gene that encodes LLP and the 5' and 3' flanking sequences of the lectin gene that encodes the phytohemagglutin-L/α-amylase inhibitor. When this chimeric gene was expressed in transgenic tobacco, a series of polypeptides Mr 10,000-18,000Da was expressed that crossreacted with antibodies to the bean α-amylase inhibitor. Most of these polypeptides bound to pig pancreas amylase affinity column confirming that the gene product was an α-amylase inhibitor. An extract of the seeds of the transformed tobacco plants inhibits pig pancreas a-amylase activity as well as the α-amylase present in the midgut of *Tenebrio molitor*.

To find out if the expression of the chimeric gene was developmentally regulated in tobacco seeds, extracts were made of seeds obtained from transformed tobacco plants at different stages of development, 9-30dpa. The extracts were analysed by SDS-PAGE and immunoblot and for inhibitory activity. The α-amylase inhibitor activity could be detected between 12-15 dap and reached a maximum level by day 20.

The gene encoding the α-amylase inhibitor protein is known to be proteotically processed to polypeptides of 15,000 to 18,000Da after its synthesis in the endoplasmic reticulum as a polypeptide of 35,000Da (Bollini *et al.*, 1986; Ceriotti *et al.*, 1989). An important consideration concerning expression of this gene in plants is the fact that the α–amylase inhibitor is a vacuolar protein. The vacuoles of leaves (Boller *et al.*, 1979) and also of other organs are rich in endoproteolytic activity, and so it will be susceptiple to proteolytic degradation. Whereas the vacuoles of seeds have low activity and therefore most suited for limited proteolytic processing of proteins. Further work is required to show that the α-amylase inhibitor retards larvae growth.

Introduction of α-amylase inhibitors in other plants could be a strategy to protect the seed against seed eating larvae. Because the pH optimum for the formation of the protein complex

between α-amylase inhibitor and amylase is five to six the inhibitor may work better in Coleoptera than against Lepidoptera. The former has an acidic pH, whilst the latter has a basic pH in their midgut (Altabella and Chrispeels, 1990).

Johnson *et al.* (1989) recently showed that the expression of potato or tomato proteinase inhibitor II in tobacco plants severely inhibited the growth of *Manduca sexta* larvae

1.14 Nutritional and metabolic effects of enzyme inhibitors

When considering the transfer of foreign genes, such as α-amylase inhibitors into food crops, it is of paramount importance to realise the possible toxicological effects that the resulting gene product may have on the consumer. α-amylase inhibitors have the potential to cause nutritional disorders and toxic effects when ingested by animal and humans as components of plant foods. When fed to experimental animals such as rats, mice and chickens, raw soybean and other legume products which contain high levels of proteinaceous inhibitors can cause adverse physiological effects (Liener and Kakade, 1980).

Plant proteinaceous inhibitors can inactivate the digestive enzymes of humans if they reach the small intestine unaltered (Richardson, 1991). Realistically this is only a problem in infants and patients with impaired peptic or gastric functions. Fortunately most methods of cooking and processing will destroy the inhibitors (Liener and Kakade, 1980).

Additionally, the insect α-amylase inhibitor isolated from barley flour is the main allergen associated with Bakers asthma disease (Barber *et al.*, 1989).

1.15 Medical applications

Puls and Keup (1973) have studied the influence of an amylase inhibitor preparation from wheat on blood glucose and insulin levels in human volunteers, rats and dogs. They showed that hyperglycaemia and hyperinsulinaemia resulting from raw starch loading could be reduced dose-dependantly by the addition of the inhibitor to the starch load. Suggestion being that α-amylase inhibitors could be used as therapeutic agents to reduce postprandial hyperglycaemia and hyperinsulinaemia in patients suffering from diabetes mellitus, obesity, hyperlipoproteinemia and related diseases.

1.16 Anti pathogen genes from non plant origin

The parasporal inclusion (Angus, 1954) bodies of certain strains of *Bacillus thuringiensis* demonstrate toxicity to a wide range of insects including dipteran, lepidopteran and coleopteran pests (Dulmage, 1981). More recently six novel genes encoding proteins toxic to

a number of plant parasitic nematodes species have been clones and characterised (Schwab *et al.*, 1992). The specificity and enviromentally benign nature of these toxins make them attractive alternatives to conventional chemical pesticides. Crystal/spore preparations of B.t. have been used as commercial insecticides in products such as Dipel^R (Abbot Laboratories) for many years. The genes encoding these toxins have been extensively investigated with regard to the engineering of pest resistant crops. Several genes encoding insect control B.t. proteins have been isolated and sequenced, and transferred into important crop plants such as tomato (Fischoff *et al.*, 1987), tobacco (Vaeck *et al.*, 1987) and cotton (Perlak *et al.*, 1990) to confer insect resistance .

Initial expression of wild type B.t genes in plants was poor and did not provide commercial levels of insect control in the field. Recently B.t. expression was optimised by significant modification of B.t. structural gene encoding sequences, which has led up to 1000-fold increases in plant expression of these genes. Perlak *et al.* (1991) hypothesised that a gene with a sequence adapted for a Gram-positive prokaryotic may not be the appropriate coding sequence for efficient plant expression. Taking into account additional factors such as codon usage in plants, potential secondary structure of mRNA, and potential regulatory sequences, synthetic genes encoding for B.t were designed which were expressed in plants at a higher level. This approach has been succesfully applied to at least four distinct types of B.t. genes. Plants expressing the higher levels of B.t. proteins obtained with these modified genes have been field tested and show commercial levels of insect control.

Fischoff (1992) reports that cotton plants expressing lepidopteran active B.t. genes from B.t. var. kustacki were tested in the field in 1990 and 1991 at six locations across the U.S. cotton belt. At five of these locations the plants were subjected to either natural or artificial infestation by cotton bollworm and tobacco budworm (the Heliothis complex), the primary lepidopteran pests of U.S. cotton. In Arizona the plants were infested with pink bollworm, the primary lepidopteran pest in the Southwest. In all locations in both years the B.t. cotton plants performed as well as or better than control plants treated with chemical insecticides (pyrethroids), the current standard for lepidopteran control.

Given the commercial level of performance of B.t. cotton plants in the field, future field tests will focus on two issues. Firstly, a total package of integrated pest management for insect pests in cotton centred around B.t. cotton will be developed. Secondly, appropriate insect resistance management strategies to delay or prevent the potential development of insect resistance to B.t. will be developed and implemented (Fischoff, 1992).

Figure 1.1 Developing fruiting bodies of *Coix lachryma-jobi.*

Additional trials with transgenic Russet Burbank potato plants expressing of the gene from B.t. var. tenebrionsis illustrated resistance against the Colorado potato beetle.

1.17 *Coix lacryma-jobi* (Job's tears)

Coix lacryma-jobi is native to southeast Asia and was cultivated very early in India (1000-2000 B.C.) and presently in South East Asia, Japan and Southern Brazil (Figure 1.1). Several species of *Coix*, a genus of monoecious grasses (Graminaceae) with unisexual flowers, are used for human food or animal forage in parts of southeast Asia.

Little crop improvement work has been done on the crop, although the grain is highly nutritious. Little is known about its origin, although it is suggested that it is closely related to maize. The grain is used as a source of ornamental beads, made into bear and tea or eaten like rice. The advent of rice may have inhibited further development of *Coix lachryma-jobi* as a crop plant although it is seen as a crop with consierable potential. The ratio of the bran to milled grains is higher than in rice, and developing a technology to use *Coix lachryma-jobi* as seed bran for foodstuff or cattle food is underway in Japan (Ohtsubo *et al.*, 1989).

1.18 The *Coix* α-amylase inhibitor

Ary *et al.* (1989) purified an α-amylase inhibitor from the seeds of *Coix lacryma-jobi* using ammonium sulphate precipitation, affinity chromatography on Red Sepharose and reverse-phase HPLC. The inhibitor consisted of two major isomers, each was found to be a dimer of closely similiar or identical subunits of approximately 26,000Da molecular weight, and linked by interchain disulphide bonds. These isomers had similiar amino acid compositions. The major isomer was assayed for inhibitor activity against α-amylases from *Bacillus subtilis*, porcine pancreas, human saliva, *Aspergillus oryzae* and barley malt, and found to be non-inhibitory. Interestingly, the major isomer did inhibit α-amylase from *Locusta migratoria nigratoriodes* (African migratory locust). The partial amino acid sequence of the major isoform revealed a high homology with partial and complete amimo acid sequences of endochitinases from a number of sources including barley seeds, and leaves of tobacco, potato and beans. Endochitinase activity was demonstrated by following the release of radioactivity from tritiated-labelled chitin.

The novel combination of enzymatic and inhibitor functions suggests a possible role of the protein as a concerted protection mechanism of the seed against insect feeding and fungal attack. The bifunctional nature of enzyme and enzyme inhibitor activity appears to be the first of its kind and the *Coix* inhibitor cannot be assigned to an existing family of the proteinaceous inhibitors (Richardson, 1991).

1.19 Coixins

Coixins are the major seed storage proteins of *Coix* (Ottabani *et al.*, 1990). They account for more than 60% of the total endosperm protein and share extensive homology to zeins, the seed storage protein in maize. Coixins are synthesised by membrane bound polyribosomes and assembled into protein bodies as are the zeins (Leite *et al.*, 1991). Following differential solubility studies, coixins are separated into two fractions, α- and γ- coixins (Leite *et al.*, 1991; Targon, 1992).

In maize, the α-zeins make up about 85% of the protein body proteins, the remaining 15% is made up of 3 classes of protein β, γ, and δ–zeins. The α-zein gene family consists of some 75 genes and can be divided into 4 subfamilies, based on nucleotide sequence of the genes and the deduced amino acids of their proteins. These 4 subfamilies have been called SF1, 2, 3 and 4 (Park, 1980).

The α-zeins are clustered in 3 chromosomes, chromosome 4, 7 and 10. Gene members of the different α-zein subfamilies share at least 65% nucleotide sequence similiarities between subfamilies and encode structurally, biologically, and functionally related α-zein proteins (Liu and Rubenstein, 1992). The α-zein SF4 gene cluster contains five genes all of which have the same transcriptional orientation. About eight repetitive elements were identified within this α-zein SF4 gene cluster. The zein genes are highly active, during peak synthesis 80% to 90% of the endosperm protein synthesis is zein. There are no introns in the zein genomic sequence althougth researchers have located a putative TATA box, a putative CAT box and two polyadenylation signals.

A number of zein specific transcripts, named precursor mRNA's, exist in the maize endosperm, in addition to the final sized mRNA of 900 bases. The precursor RNA's range in size from 1800 bases to over 3800 bases. It is assumed that the precursor mRNA's are transcribed to include regions flanking the zein genes.

Transcriptional analysis of the zein genomic sequences has revealed 2 *in vitro* active promoters (P_1 and P_2), one produces an RNA equivalent to the 1800 base precursor RNA and the second a 900 base mRNA (Langridge and Feix, 1983; Hu *et al.*, 1982). It has been suggested that the large zein specific mRNA's represent part of a complex expression mechanism employed to obtain intensive zein synthesis. P1 is found 1000 bases upstream of the zein gene start and P2 lies directly before the gene. The N terminal region of the zein gene position 1-128 includes the signal peptide and has been found to agree closely with published cDNA. The region 5' to the zein gene is on average 67% A+T and contains only short reading frames, none of which are preceeded by TATA or CAAT.

The cDNA clone encoding the γ-coixin has been isolated from a cDNA library constructed from polysomal mRNA isolated from the immature *Coix* endosperm (Leite *et al.*, 1991). The nucleotide sequence of this clone (pBCX22.5) revealed a 27-nucleotide 5' untranslated sequence followed by a 600-nucleotide open reading frame and a 3' noncoding sequence of 207 nucleotides. Three putative polyadenylation signals, AATAAT, AATAAA and AATGAA, were found 162, 98 and 29 nucleotides respectively from the poly(A) tail.

Translation of the cDNA clone predicted a polypeptide of 199 residues,which was 77% homologous to the 27kD γ-zein and 67% homologous to the 16kD γ-zein. The first 19 amino acids of γ-coixin share 95% homology with the 27 and 16kD γ-zeins signal peptides and may represent the signal peptide of γ-coixin (Leite *et al.*, 1991). Additionally, the γ-coixin mature protein contains 180 amino acids, corresponding to a molecular mass of 19,600Da. The N terminal tail of γ-coixins contains two cysteines followed by a repetitive region formed by PPPVHL motif which is similiar to γ-zeins. The differences in the molecular masses of 27 (21.8) kD γ-zein, and 22 (19.6) kD γ-coixin appear to be due to differences in the number of PPPHVL repeats.

1.20 Aims and objectives

The aim of this project was to isolate the gene encoding a bifunctional α-amylase inhibitor/endochitinase from the seeds of *Coix lacryma-jobi* using a multifunctional approach. It was envisaged, that once the gene had been isolated and characterised it may have been used to construct transgenic plants to confer insect resistance. Additionally, the gene was of interest academically, as few genes encoding bifunctional inhibitors have been characterised.

Three strategies were adopted for isolation of this gene;

1. Immunoscreening of a *Coix* seed λgt11 cDNA expression library with polyclonal antibodies raised against an endochitinase protein.

2. Production of α-amylase inhibitor antibodies, raised against a *Coix* α-amylase inhibitor fusion protein. It was envisaged that once antibodies were available, immunoscreening of a *Coix* seed λgt11 expression cDNA library would result in isolation of α-amylase inhibitor/endochitinase cDNA clones.

3. Amplification of the α-amylase inhibitor coding sequence from *Coix* genomic DNA, cDNA and a seed cDNA library.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials.

All chemicals and biological reagents, with the exception of those noted below, were from the Sigma Chemical Company Ltd or BDH Ltd and were "AnalaR" or the best grade obtainable.

All restriction endonucleases, DNA modifying enzymes, X-Gal and IPTG were supplied from Northumbrian Biolabs or Boehringer Mannheim.

T4 DNA ligase, calf intestinal alkaline phosphatase and caesium chloride were from the Boeringer Corporation (London) Ltd, Lewes, East Sussex.

5-bromo-4-chloro-3-indoyl-β-D-galactosidase (X-Gal), Tris (hydroxy methyl), aminomethane, Agarose, LMP Agarose were from Bethesda Research Labs (U.K.) Ltd. Cambridge, U.K.

BBL trypticase peptone was from Becton Dickinson and Co., Cockeysville, M.D., U.S.A.

Bacteriological agar and yeast extract was from Oxoid, Basingstoke, Hampshire, U.K.

Sodium Chloride was from Reidal-de-Haen, D 3016, Seeize 1, Germany.

Nitro-cellulose filters were from Schleicher and Schuell, Postfach 4, D-3354, Dassel.

Ministart filters were from Sartorius GmbH, Post-fach 3243, D-3400 Gottingham, West Germany.

3MM paper was from Whatman Ltd., Maidstone, Kent, U.K.

High vacuum grease was from Dow Corning S.A., Seneffe, Belgium.

Poloroid 667 film was from Poloroid (U.K.) Ltd., Hertfordshire.

All radiochemicals were from Amersham International plc.

2.2 Bacterial strains.

All bacterial strains used during the course of this work are outlined below.

E. coli

DH5α	*end*A1, *hsd*R17, *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, Δ(*lacZYA-arg*F) U169, φ80, d*lac*ZΔM15. BRL (1986).
XL1-Blue	*end* A1, *hsd* R17 ($r_k^- m_k^+$), *sup* E44, *thi*-1, λ⁻, *rec*A1, *gyr* A96, *rel* A1, *Δ(lac)*, [F' *pro*AB, *lac*I^q, *lac*ZΔM15, Tn10(Tet^R)]
Y1088	E.coli Δ(*lacU169*), *sup*E, *sup*F, *hsd*R (r-, m+), *met*B, *rp*R, *ton*A21, [*proC*::Tn5] (pMC9)=pBR322-*lac*I^Q Davis *et al.* (1980)
Y1090	*E.coli* Δ(*lacU169*), *pro*A$^+$, D(*lon*), *ara*D139, *str*A, *sup*F, [*trpC22*::Tn10(tet^r)], (pMC9), *hsd*R(rk$^+$, mk$^+$) Davis *et al.* (1980)

2.3 Plasmids.

Described below are the plasmids used during the course of this work.

Plasmid	Comments	Reference
pUC18/19	amp^R	Viera and Messing (1982)
pBluescript	amp^R, with T7 promoter	Stratagene
λgt11	amp^R	Young and Davis (1983)

λEmbl3		Frischauf *et al.* (1993)
pGEX3X	amp^R	Smith and Johnson (1988)

2.4 Media and growth conditions.

Bacterial strains were routinely grown in L-Broth

or on L-agar at either 37^0C (*E.coli*) or 42^0C (phage hosts).

L-Broth:	Tryptone	10g
	Yeast extract	5g
	NaCl	5g
	Distilled water	to 1 litre
	(For L-agar 15g of agar were added)	

The media was then autoclaved.

If agar plates were required for blue/white colour selection when using pUC, pBluescript or λ phage, X-Gal was made up as a 2% solution in dimethylformamide and 200μl added for every 100ml autoclaved agar.

For purification of phage DNA, λgt11 clones were grown on NZCYM media:

NZCYM Media:	N_2 amine	1g
	NaCl	0.5g
	Yeast	0.5g
	Casamino acids	0.1g
	$MgSO_4.7H_2O$	0.2g

The volume was made up to 100ml with dH_2O and the pH adjusted to pH 7.5 with sodium hydroxide and then autoclaved.

(For NZCYM agar 1.5g of agar was added)

When antibiotic selection was required, stock solutions were prepared, filter sterilised and the appropriate volume added to cooled, autoclaved media as detailed below.

Antibiotic conc.	Stock solutions (mg/ml)	Solvent	FC* (μg/ml)
Ampicillin (Amp)	50	H_20	50
Kanamycin (Km)	25	H_20	25
Tetracycline (Tc)	12.5	EtOH	10

*Final concentration

2.5 Sterilisation procedure.

All glassware, plasticware and other equipment required for aseptic manipulation was autoclaved for 15 minutes at 120^0C, 15psi. Solutions were autoclaved or filter sterilised through 0.2μm filters before use.

2.6 Storage of bacteria.

Bacterial cultures were stored on agar plates sealed with nescofilm for no longer than 6 weeks in a 4^0C refrigerator. For long term storage, freshly steaked out colonies were inoculated into 10ml of the appropriate growth broth with selection and incubated at the correct growth temperature overnight. The resulting culture was pelleted, then resuspended in 1ml of L-broth with 1ml of 80% glycerol and stored at -80^0C.

2.7 Plant material.

Coix lachryma-jobi seeds were a gift from M. Ary, Rothamstead Experimental Station, Harpenden (U.K.). *Coix* plants were grown in the greenhouse and hulls were marked on their day of flowering. Aliquots of seeds were collected every third or fourth day until full maturation of seeds (28th day).

2.8 Isolation of plasmid DNA.

2.8.1 Mini-preparation.

Small amounts of plasmid DNA were prepared essentially as described in, Sambrook *et al.* (1989).

An overnight culture of the bacteria was grown with the appropriate antibiotic selection and 1.5ml aliquoted into a sterile Eppendorf tube. The suspension was centrifuged for 1-2 minutes at low speed in a MSE Microcentaur microfuge, the supernatant was removed and the pellet partially dried by inversion of the tube over tissue paper. 100μl of ice cold solution 1 was added (1% glucose, 10mm EDTA pH 8.0, 25mm Tris.Cl pH 8.0), briefly vortexed and left to stand at room temperature for 5 min. 200μl of freshly prepared solution 2 (0.2N NaOH, 1.0% SDS) was added, mixed gently by inversion, and stored on ice for 5 min. 150μl of ice cold solution 3 (11.5ml of glacial acetic acid and 28.5ml of ddH_2O added to 60ml of 5M potassium acetate) was added and mixed by vortexing followed by incubation on ice for 5 min. Chromosomal DNA and other cellular debris was pelleted by centrifugation at 13,000g for 5 min. The supernatant was removed to a sterile tube, phenol/chloroform extracted and the aqueous phase ethanol precipitated.The pellet was washed in 70% ethanol, vacuum dried and resuspended in T.E. buffer plus RNAase (200μg/ml). The DNA yield and quality was checked by U.V. spectrophotometry.

2.8.2 Large scale preparation.

2.8.2.1 High copy number plasmids.

10ml of L-broth was inoculated with a loop full of freshly streaked out bacteria and grown overnight with appropriate antibiotic selection. The following day the 10ml of culture was added to 1 litre of L-broth containing antibiotics and grown overnight with vigorous aeration. Pellets were harvested by centrifugation at 4000rpm for 10 minutes in 250ml Sorvall tubes using 6 x 500 angle head rotor in a MSE High speed centrifuge. The supernatant was discarded and the pellets washed in 20ml T.E.N. buffer (0.1M NaCl, 10mM Tris.HCl, 1mM EDTA pH 8.0) followed by centrifugation at 4000rpm for 10 minutes. The pellets were dried by inversion on tissue paper, resuspended in 10ml solution 1 (section 2.8.1) containing 5mg/ml lysozyme and left at room temperature for 30 minutes. 20ml of solution 2 (section 2.8.1) was added and the mixture kept on ice for 20 minutes, followed by the addition of 15ml of ice cold 5M sodium acetate pH 4.8, mixed well and left on ice for a further 10 min.

Lysed cells were centrifuged in a 8 X 50ml Sorvall rotor at 20,000rpm for 20 minutes at 4^0C and the supernatant transferred to sterile 30ml corex tubes (approximately 12ml per tube). 0.6% by volume isopropanol was added to the supernatant, mixed well and precipitated at room temperature for 30 minutes. DNA was recovered by centrifugation at 12,000g for 30

minutes at room temperature, washed with 70% ethanol, dried under vacuum and resuspended in 5ml of T.E. buffer. Depending on the final volume of the sample an exact amount of caesium chloride and ethidium bromide was added (CsCl 0.764g/ml, ethidium bromide 120µg/ml). Using a syringe and needle the samples were transferred to Quickseal centrifuge tubes (Beckman) which were balanced, heat sealed and centrifuged at 44,000rpm for 17-20hrs at 15^0C using a Sorvall ultracentrifuge OTDG5B. After centrifugation the tubes were examined under U.V. illumination. 2 bands were usually visible, the upper band contained chromosomal DNA, the lower band corresponded to plasmid DNA. Using a needle and syringe, the lower band was recovered and the mixture extracted several times with an equal volume of butan-1-ol saturated with CsCl to remove all the ethidium bromide. Once the sample was ethidium bromide free, it was dialysed against T.E. buffer. The final sample was checked for purity and concentration by U.V. spectrophotometry.

2.9 Transformation of bacteria

2.9.1 Preparation of calcium chloride competent *E. coli* cells.

This was a modification of the method of Mandel and Higa (1970) and was used when a large number of transformations were to be carried out, or when competent cells were made for long term storage.

50ml of L-Broth was inoculated with 1ml from an overnight culture of *E.coli* cells and incubated with shaking until an $O.D_{600}$ of 0.3-0.4 was reached (2-3hrs). The culture was chilled on ice for 10 minutes and then centrifuged at 4,000g for 5 minutes at 4^0C. The supernatant was discarded and the pellet resuspended in 1/2 the original culture volume (25ml) of an ice cold sterile solution A (50mM $CaCl_2$, 10mM Tris.HCl, pH 8.0.). The cell suspension was placed on ice for 15 minutes and then centrifuged again as before. The supernatant was discarded and the cells resuspended in 1/15 of the original volume of ice cold solution A, then dispersed into 200µl aliquots and left on ice for at least 1 hour prior to use. Cells not used immediately were stored at -80^0C with glycerol (70µl of 80% glycerol in 200 µl of cells).

2.9.2 Preparation of rubidium chloride competent *E. coli* cells.

This was a modification of the method of Kushner (1978) and was the preferred method when only a small number of transformations were to be performed as higher transformation efficiencies were obtained.

10ml (allows 6 transformations) of L-Broth was inoculated with 200µl from a fresh overnight culture and incubated until the cells reached an $O.D_{680}$ 0.4-0.5, which was approximately 2-3 hours. 1.4ml of culture in an Eppendorf was pelleted in a microcentrifuge for 30 seconds at 13,000g, at room temperature. The supernatant was discarded, and the pellet gently resuspended in 500µl of solution 1 (10mM MOPS pH7, 0.10mM RbCl). The cells were centrifuged for 15 seconds as above, resuspended in 500µl of solution 2 (100mM MOPS, pH6.5, 10mM RbCl, 50mM CaCl) and incubated on ice for 90 minutes. After a 15 second centrifugation the cells were resuspended in 150µl of solution 3 (100mM MOPS pH6.5, 100mM RbCl, 500mM $CaCl_2$) and 4µl DMSO.

2.9.3 Transformation of competent *E.coli* cells.

200µl of cells were mixed with DNA in ligation buffer or T.E. and stored on ice for 30 minutes. Controls of undigested vector, digested and religated vector were included. The cells were transferred to a 42^0C water bath for 2 minutes or to 55^0C for 30 seconds and then on ice for 5 minutes. 1 ml of L-broth was added to each tube and incubated at 37^0C for 30 minutes (tetracycline selection) or 1 hour (Ampicillin and Kanamycin selection) without shaking. 1/10 and 9/10 dilution's were plated onto L-agar with the appropriate antibiotic selection and incubated overnight at 37^0C.

Where appropriate 50µl 2% X-Gal was added to the agar allowing transformants to be selected as a result of the formation of white colonies due to insertional inactivation of the β-galactosidase gene.

2.10 General DNA Manipulations.

2.10.1 Spectrophotometric quantitation of DNA.

The DNA solution was diluted with an appropriate volume of T.E. buffer, usually a 1/20 or 1/50 dilution. The absorbance of the solution at 260 and 280nm was measured using a Beckman DU7500 Spectrophotometer. A pure DNA sample shoµld have an $A_{260/280}$ ratio of 1.8. An A_{260} of 1.0 is equivalent to a double stranded DNA concentration of 50µg/ml, RNA/single stranded DNA concentration of 40µg/ml or oligonucleotide concentration of 20µg/ml.

2.10.2 RNAase treatment of DNA samples.

A 10mg/ml stock solution of DNAase free pancreatic RNAase was made up using milli-Q water and heated at 90^0C for 10 minutes to inactivate any contaminating DNAase, this was stored at -20^0C. For treatment of DNA solutions a 1:50 dilution was added.

2.10.3 Restriction endonuclease digests.

10 X stocks of manufacturers restriction enzyme buffers were used for digests. If multiple digests were carried out One-phor-all™ buffer was used if appropriate. Plasmid DNA was generally digested with 1-2 units restriction enzymes for every 10µl digest volume, 0.1 volume of 10 X restriction enzyme buffer and the volume adjusted as required with milli-Q water. Digest's were incubated at the appropriate temperature (usually 37^0C) for the minimum of 2 hours. Digests which were to be analysed by gel electrophoresis were terminated using 0.1 volume of stop dye, which was made up as follows;

1ml	10% SDS
2ml	250mM EDTA pH 8.0
0.2ml	1M Tris.HCl pH 8.0
5ml	glycerol
1.8ml	distilled water
10mg	bromophenol blue

If the DNA was used for subcloning procedures, then the reaction was terminated by phenol/chloroform extraction.

2.10.4 Phenol:chloroform extraction of nucleic acids

DNA samples were deproteinised by adding 1/2 volume of redistilled phenol equilibrated with T.E. buffer pH 8.0 and 1/2 volume of chloroform:isoamylalcohol (24:1). The phases were mixed by vortexing for several seconds and then separated by centrifugation at 13000g in a microcentrifuge for 2 minutes. The aqueous phase was removed carefully without disturbing the interface and re-extracted with 1/2 volume chloroform:isoamyl alcohol (24:1) to remove excess phenol. The aqueous phase was recovered and ethanol precipitated.

2.10.5 Precipitation of DNA with alcohol.

0.1 volume of 3M sodium acetate and 2 volumes of 100% ethanol were added to the DNA sample, mixed by vortexing and stored at -80^{0}C for as long as possible, usually overnight. When precipitating after a mini-preparation, as sodium acetate is already present, no more was added. Precipitates were collected by centrifugation at 13,000g for 10 minutes in a microcentrifuge, or for larger samples at 12000g for 20 minutes at 4^{0}C in the sorvall RC-5B centrifuge. Pellets were washed in 70% ethanol, centrifuged as above, vacuum dried and resuspended in T.E. buffer or milli-Q water. Alternatively pecipitation was carried out using isopropanol (0.6 volume).

2.10.6 Phosphatase treatment of DNA.

By removal of the 5'terminal phosphate groups from both ends of linearized DNA using calf intestinal phosphatase, recircularization of plasmid vector DNA in the absence of insert DNA was minimised.

Up to 15μg of plasmid DNA was digested (section 2.10.3) but with excess restriction enzyme. The digests were phenol:chloroform extracted and the aqueous layer ethanol precipitated. DNA pellets were resuspended in 40μl of 10 X phosphatase buffer (0.5M Tris-HCl, 1mM EDTA, pH 8.0). 10 units of calf intestinal phosphatase were added and the reaction incubated at 37^{0}C for 30 minutes. The reaction was terminated by the addition of 4.8μl of 0.1M nitrilotriacetic acid (NTA) followed by incubation at 70^{0}C for 15 minutes. The solution was phenol/chloroform extracted, ethanol precipitated and the final DNA pellet resuspended in milli-Q water at approximately 0.1μg/ml.

2.10.7 Ligation of DNA fragments.

DNA fragments with compatible cohesive or blunt ended termini generated by digestion with resriction enzymes were ligated together by the action of T4 DNA ligase.

A ratio of 3:1 insert to vector DNA was used for each reaction. 0.1 volume 10 X ligase buffer (0.66M Tris.HCl pH 7.5, 50mM $MgCl_2$, 50mM DTT) and 2 units of T4 DNA ligase were added. For blunt ended ligations the amount of enzyme was increased to 5 units. The ligation mixture was incubated overnight at 15^{0}C or 2 hours at room temperature. An appropriate aliquot of the ligation mix was used to transform competent bacterial cells.

2.10.8 Dialysis of DNA solutions.

Dialysis tubing was boiled for 10 minutes in 100ml of 2% (w/v) sodium carbonate and 0.4 ml 250mM EDTA, rinsed 5 times with distilled water and boiled for a further 10 minutes. The tubing was stored at -4^{0}C in distilled water with 1mM sodium azide to prevent microbial

growth. The DNA solution was transferred to the tubes, each end was folded and clipped making sure space was left at one end. The samples were dialysed with 2 litres of TE buffer at 4^0C with stirring for 24 hours. The buffer was changed after 2 and 6 hours.

2.10.9 Preparation of denatured salmon sperm DNA.

Denatured salmon sperm DNA was incorporated into prehybridisation and hybridisation buffers when probing Southern blots and colony screens, to prevent non-specific hybridisation of labelled DNA. Salmon sperm DNA (10mg/ml) was dissolved in sterile distilled water by stirring overnight at room temperature. The DNA was sheared by extruding three times through an 18 gauge needle and denatured by heating to 100^{0}C for 10 minutes. The salmon sperm DNA was allowed to cool and stored in 0.5ml aliquots at -20^{0}C. Prior to use an aliquot of salmon sperm DNA was heated in a boiling water bath for 10 minutes and placed on ice.

2.10.10 Agarose gel electrophoresis.

Agarose gel elecrophoresis was used in order to identify and purify DNA fragments. The percentage gel used depended on the size of the DNA fragment under study. For most applications 0.7% (w/v) agarose gels were used which are recommended for the separation of DNA fragments in the range of 10-0.8kb (Sambrook *et al.*, 1989)

Agarose was dissolved in Tris-borate buffer (89mM Tris-borate, 89mM boric acid, 2mM EDTA) or Tris-acetate buffer (40mM Tris acetate, 1mM EDTA pH 7.7.) by heating in a microwave oven for 2-3 minutes. The gel was poured into an appropriate gel former and when set the gel former and comb were removed and the gel immersed into a gel tank containing the appropriate buffer. Samples were mixed with 0.2 vol of gel loading buffer (25mM Tris-HCl pH 8.0, 50mM EDTA, 1% SDS, 7.5% ficoll, 0.25% bromophenol blue) and loaded into wells and electrophoresed for 4-5 hrs at 100v or overnight at 30V. Ethidium bromide was added to both buffer and gel at a concentraton of 0.5μg/ml in order to visualise the DNA bands. DNA was visualised under long wave ultraviolet light (320nm) and photographed using a red filter and Polaroid 667 film.

2.10.11 Preparation of DNA gel size standards

λ Pst I and DNA markers were prepared as below;

33μl λ DNA (10μg), 10μl appropriate buffer, 57μl distilled H_20, 60 units restriction enzyme were incubated at 37^{0}C overnight. The reaction was stopped by the addition of 18μl of 6 X stop dye and aliquots used alongside samples for DNA electrophoresis.

Fragment sizes for the λDNA/ Pst I digests were as follows;

(base pairs);

14,050, 11,490, 5,070, 4,750, 4,510, 2,838, 2,556, 2,460, 2,443, 2,140, 1,968,1,700, 1,158, 930, 805, 514, 470, 450, 340.

2.10.12 DNA fragment isolations.

2 procedures were used for fragment isolations.

2.10.12.1 Method 1.

DNA samples were loaded onto an agarose gel and electrophoresis carried out as described in section 2.10.10. The DNA was visualised under U.V light and the appropriate fragment cut out using a sterile scalpel removing as little agarose as possible. The DNA containing fragment was frozen at -80^{0}C for 15 minutes centrifuged through siliconized glass wool for 10 minutes at 13,000g in a micro centrifuge and extracted with 100μl of chloroform/isoamylalcohol (24:1). To the aqueous phase 1/10 vol. of 3M Na.0Ac, 0.1M Mg.0Ac pH5.2 and 2 vol. ethanol was added and the DNA precipitated at -80^{0}C for 30 minutes or more. The pellet was collected by centrifugation at 13000g for 10 minutes, washed in 70% ethanol, vacuum dried, and resuspended in T.E. buffer.

2.10.12.2 Method 2.

This method utilises silica fines, a Tris-acetate buffer system along with low melting point agarose for gel formation.

After electrophoresis of the sample DNA, the required DNA fragment was isolated (section 2.10.12.1), melted at 70^{0}C in a water bath for 15 minutes and mixed with 1ml of a NaI/Na_2SO_3 solution. 5μl of silica fines (gift of Dr. Robinson, University of Durham, Durham.) was added and the mixture incubated at room temperature for 30 min. The solution was centrifuged at 13,000g for 2 consecutive spins at 15 and 2 seconds removing the supernatant between spins. The silica fines were resuspended in 1ml of 70% ethanol by vortexing and then centrifuged again as above. The silica fines were incubated in 20μl of T.E. buffer at 37^{0}C for 30 min followed by a 15 sec. centrifugation at 13000g. The resulting supernatant was removed to a fresh Eppendorf and the yield of DNA recovered quantified by U.V. spectrophotometer.

2.10.13 Preparation of radioactively labelled probes

2.10.13.1 Oligonucleotide End Labelling.

The following were incubated at 37⁰C for 30 minutes in an Eppendorf:

35μl dH_2O

25-50ng DNA

10μl 10x kinase buffer

1μl (5 units) T4 kinase

5μl γ^{32}P-ATP

2.10.13.2 Random Primer Reactions.

An Amersham multiprime kit was used for these reactions. Approximately 30-50ng DNA in a total volume of 28μl was boiled for 5 minutes and then transferred to ice for 2 minutes. 10μl of labelling buffer, 5μl of primers, 5μl ^{32}P-dCTP and 2μl of Klenow enzyme were added and incubated at 37⁰C for 2-3 hours or room temperature overnight.

2.10.13.3 Purification of radioactivity in nucleic acids.

The labelling reaction was stopped by the addition of 10μl of Stop-Dye (60mM EDTA, 100mg/ml blue dextran, 1mg/ml xylene cyanol) and the mix loaded onto a Sephadex G50 column equlibrated in T.E. pH 8.0. The probe which elutes with the blue dextran dye was collected and 5μl counted in a scintillation counter. Approximately 5×10^5 to 1×10^6 dpm/ml of hybridisation mix was used.

2.10.14 *In situ* hybridisation of bacterial colonies

The method used was that described by Sambrook *et al.* (1989).

A gridded nitrocellulose filter was layed on top of an L-agar plate containing the appropriate antibiotic selection. Putative transformants were transferred to it using sterile cocktail sticks. Master plates were also made. The plates were incubated at 37⁰C overnight and both filter and master plate were marked in three identical positions in order to identify positive colonies. The nitrocellulose filters were removed and the cells lysed by placing on Whatmann 3MM paper impregnated with 10% SDS for 2 minutes and then onto Whatmann 3MM paper which had been soaked with denaturation buffer. After 5 minutes the filters were removed to 3MM impregnated with neutralization buffer for a further 5 minutes. The filters

were air dried for 30 minutes and then baked in a vacuum oven at 80⁰C for 1 hour. To prepare the filters for hybridisation they first were wet in 6 x SSC and then washed for 1-2 hours at 65⁰C in prewashing solution (50 mM Tris.Cl pH 8.0,1M NaCl, 1mM EDTA pH8.0, 0.1% SDS.). The filters were then immersed in prehybridising solution (5 x Denhardts 5 x SSC, 0.1% SDS, 0.1% pyrophosphate, 100μgml^{-1} denatured salmon sperm DNA) and incubated with shaking for 4-6 hours at 65⁰C. The denatured labelled probe was added and incubated at 65⁰C overnight. The filters were washed for 5-10 minutes in 2 x SSC, 0.1% SDS at room temperature. If background was still high the filters were washed twice in 0.1% SSC, 0.1% SDS.at 65⁰C, then allowed to air dry.

2.10.15.1 Transfer of DNA to nitrocellulose membranes

The method was a modification of that of Southern (1975).

A large DNA-containing agarose gel was run (section 2.10.10) and photographed with a ruler at the side of the gel. If transfer involved large fragments then the gel was soaked in 1% HCl for 15 minutes (depurination) and the gel rinsed with dH_20 prior to incubations with denaturation buffer (1.5M NaCl, 0.5M NaOH) for 45 minutes followed by incubation for 45 minutes in neutralization buffer (1.5M NaCl, 0.5M Tris.Cl pH 7.0), the gel was then rinsed in 20 x SSC (3M NaCl, 0.3M sodium citrate) and blotted onto nitrocellulose overnight as in Sambrook *et al.* (1989). The nitrocellulose was removed from the blotting apparatus and baked between two sheets of Whatman 3MM for 1 hour at 80⁰C under vacuum and stored at room temperature until required.

2.10.15.2 Hybridisation of labelled probes to Southern blots.

The blot was incubated in 25ml of prehybridising solution (6 X SSC, 5 X Denhardts, 0.5% SDS containing 0.5ml 1mg/ml solution of denatured salmon sperm DNA) for 1 hour at 65⁰C. The blot was then incubated in a fresh 25ml of prehybridisation buffer containing the denatured probe and hybridised overnight at 65⁰C. Following hybrididation the filter was washed with 50ml 2 X SSC at 65⁰C for 15 minutes twice, followed by a 15 minute wash at 65⁰C in 2X SSC, 0.1% SDS. If the background was still high, a final wash with 50ml 0.1 X SSC at 65⁰C was carried out.

2.11 Autoradiography.

Autoradiograpy was used to detect ^{32}P-labelled nucleic acids on nitrocellulose filters. The nitrocellulose filter was wrapped in cling film and the following procedure carried out in a dark room under a red safe-light.

X-ray film (Fuji RX) was preflashed and placed "flashed"-side down on the filter. Both were placed in a cassette containing an intensifiying screen with the screen nearest the film. The autoradiograph was stored at -70^{0}C for at least an hour, and up to a month if there were no detectable counts on the film. The X-ray film was developed in Kodak X-Omat developer for 5 minutes, rinsed in water and immersed in Kodak fixer for 5 minutes, rinsed and air-dried.

2.12 Genomic DNA isolation from plant tissue.

The following method is based on the Dellaporta plant DNA miniprep technique (Dellaporta *et al.*, 1983) This method has the advantage of isolating relatively good yields of DNA from small amounts of starting material (approximately 40μg DNA from 1-2g of tissue) additionally, the speed of isolation allows relatively undegraded material to be retrieved. The miniprep method starts of with approximately 2g of leaf tissue, for larger preparations the procedure was scaled up accordingly. Leaf tissue was ground to a fine powder in a pestle and mortar containing liquid nitrogen, then transferred to a centrifuge tube containing 15ml extraction buffer (100mM Tris/HCl pH8.0, 50mM EDTA pH 8.0, 500mM NaCl and 10mM β-mercaptoethanol) and 1ml 20% SDS, shaken vigorously and incubated at 65^{0}C for 20-30 minutes. Following incubation on ice with 5mls of 3M CH3COOK, pH 4.8, the solution was centrifuged at 9,000rpm for 20 minutes and the supernatent passed through mira cloth into a clean centrifuge tube. The mix was phenol/chloroform extracted and precipitated with 10ml isopropanol at -80^{0}C overnight. Following centrifugation at 9,000rpm for 20 minutes the precipitant was resuspended in 0.7mls resuspension buffer (50mM TrisHCl pH8.0, 10mM EDTA) and incubated at room temperature for 30 minutes. The solution was transferred to an Eppendorf and centrifuged at 12,000 rpm for 10 minutes, the supernatent was removed, phenol:chloroform extracted and ethanol precipitated. The pellet was resuspended in 50μl of resuspension buffer II (10mM Tris/HCl pH8.0, 1mM EDTA) and an aliquot electrophoresed on an agarose gel to check the yield.

2.13.1 Plant RNA Preparation.

Approximately 1-2g of plant tissue was ground to a fine powder under liquid nitrogen using a mortar and pestle and then transferred to eppendorfs containing 0.5ml of extraction buffer (50mM Tris.HCl, 150mM LiCl, 5mM EDTA, 5% SDS pH 9.0) and incubated on ice for 5 minutes. The mixture was phenol/chloroform extracted twice followed by a chloroform:isoamyl alcohol extraction. The aqueous phase was removed and sterile 8M LiCl was added to a final concentration of 2M LiCl, precipitation occured at -20^{0}C overnight. The precipitate was collected by centrifugation at 12,000rpm and the pellet resuspended in 600μl of sterile dH_20, 200μl of 8M LiCl was added and precipitated as before. The total RNA was

ethanol precipitated at -20^{0}C for 2 hours, vacuum dried briefly taking care not to totally dry the pellet and resuspended in 10-20µl of 0.5M NaCl. An aliquot of the sample was run on an RNA denaturing gel to quantify the yield. RNA was stored at -80^{0}C.

2.13.2 RNA Gel electrophoresis and Northern blotting

RNA samples were electrophoresed on denaturing 1% agarose gels containing formaldehyde according to the method given in Berger and Kimmel (1987).

1g agarose in 62.5 mls of dH_20 was dissolved by heating for 3 minutes in a microwave oven, and added to 20mls of 5x running buffer (0.2M MOPS pH7.0, 50mM NaOAC, 10mM EDTA pH8.0) and 17.5 ml formaldehyde (12.3M). Once cooled this was poured into the appropriate gel former, prior to loading of the samples the gel was prerun at 60V for 30 minutes to remove formaldehyde from the wells. Approximately 10µg of RNA in a total volume of 10µl was added to 30µl of denaturing solution (500µl deionised formamide, 200µl 5 X running buffer, 150µl filtered formaldehyde and 150µl ddH_2O) and the mix incubated at 55^{0}C for 15 minutes. When the samples were cool, 8µl of 6 X loading buffer was added (50% glycerol, 1mM EDTA pH8.0, 0.4% bromo-phenol blue, 0.4% xylene cyanol). The samples were loaded onto the gel and electrophoresed at 60V for 5-6 hours in a fume hood. The gel was stained for 2-5 minutes in ddH_20 containing 5µg/ml ethidium bromide. The gel was destained overnight in sterile dH_20, with frequent changes, until the RNA bands were visible. The RNA was transferred overnight onto Hybond-N nylon by capillary action in 20 X SSC as in Sambrook *et al.* (1989). When transfer was complete the membrane was air dried for 30 minutes and then the RNA crosslinked to the nylon by exposure to ultraviolet light for 5 minutes.

2.14.1 *In vitro* translation of PolyA$^+$ RNA.

All *in vitro* translation experiments were carried out using a rabbit reticulocyte lysate kit, Amersham N.90, and L-^{35}S methionine as the labelled amino acid. The lysate is a general purpose reagent for protein synthesis and contains a "cold" amino acid pool. In addition potassium and magnesium ions are present at concentrations optimised for a wide range of mRNA's. *In vitro* translations were set up as follows: 40µl lysate, 1µl placental RNAase inhibitor, 25units/µl, 72µCi Trans ^{35}S label and 5µl of Poly A$^+$ RNA from seeds of *Coix lacryma-jobi* were mixed in a Eppendorf. This was incubated at 30^{0}C for 2.5 hours, followed by 37^{0}C for 15 minutes in order to hydrolyse amino-acyl-tRNA complexes.

Following incubation 100mM unlabelled methionine was added to 5% and 0.5M Tris-HCl pH 6.8 was added to a final concentration of 60mM. Aliquots were removed and stored at

-20^{0}C until required for loading alongside immunoprecipitation samples when analysed by SDS-PAGE.

2.14.2 Immunoprecipitation of *in vitro* translation products.

In vitro translation were set up as before and an aliquot removed and mixed with 18.5μl of 2 X immunoprecipitation buffer, 20μl of cold 100mM methionine and 180μl of immunoprecipitation buffer with detergent. This was mixed with the α-amylase inhibitor antibodies at a dilution of 1/500 and incubated at room temperature overnight. 50μl of protein A Sepharose (1 mg/ml) equilibrated in immunoprecipitation buffer was added to this mix and incubated at room temperature for 1 hour. The antigen-antibody complex which binds to the Sepharose was pelleted by centrifugation at 12,000rpm for 3 minutes. The pellet was washed 3 times with ice cold immunoprecipitation buffer + detergent followed by a final wash without the detergent as a precaution against the generation of abnormal SDS-PAGE migration patterns. The pellet was resuspended in 30μl of SDS sample buffer and heated to 100^{0}C for 3 minutes to release the labelled proteins. Protein-A-Sepharose beads were removed by centifugation as before and and aliquots of each reaction were electrophoresed on a 12.5% SDS-PAGE with a 5% stacking gel at a constant current of 40mA (maximum voltage 250V). The gel was fixed overnight in 10% isopropanol and 10% acetic acid and then the radiation enhanced by incubating in 20% isopropanol, 4% glycerol and 1M sodium salicylate. The gel was vacuum dried and exposed to X-ray film as described in section 2.11.

2.15 General protein manipulations.

2.15.1 SDS-PAGE.

The method used was similiar to that of Laemmli (1970). Depending on the requirements of the experiment either the BioRad Protean II Xi cell or the BioRad mini protean II protein gel apparatus were used. Gel apparatus was assembled according to the manufacturers instructions. All glass plates were cleaned with detergent followed by 100% ethanol prior to use.

A 12% running gel with 6% stacking gel was prepared as follows;

	12% running gel (ml)	6% stacking gel (ml)
30% acrylamide	24ml	1.5ml

(29.2% acrylamide

0.2% bisacrylamide)

Buffer	30ml(A X2)	3.75(B X2)
Ammonium persμlphate (2%)	2ml	0.25ml
H_2O	4ml	2.0
TEMED	60μl	20μl

(Buffer A X2 contains 0.75M Tris/NaOH pH 8.8, 0.2% SDS

Buffer B X2 contains 0.25M Tris/HCl pH6.8, 0.2% SDS).

Mini-protean gels were electrophoresed at 150mA constant current, 150V for 2 hours. The larger gels were electrophoresed at 30V overnight.

2.15.2 Western blotting.

Protein samples were electrophoresed in SDS-PAGE in the normal manner, loading Sigma high and low molecular weight markers alongside the samples.

The gel was placed in 100-200ml of transfer buffer (25mM Tris, 192 mM glycine, 20% methanol) for 15 minutes (0.75mm gel) or 60 minutes (3-6mm gel). The gel was assembled in the transfer apparatus and transfer was carried out onto nitrocellulose Hybond-C using a Bio-Rad Trans Blot^{TM} at 30V/40mA overnnight or 100v/150 mA for 1-2 hours. To check the transfer of the proteins onto the nitrocellulose the membrane was stained in Ponceau S (0.2% Ponceau S in 3% TCA) for 5 minutes and then destained in 1% Acetic acid. If the transfer was adequate the the membrane was photographed.

The membrane was blocked in 1% gelatin in TBS (10mM Tris.HCl pH 7.2, 170mM NaCl, 30mM KCl) with shaking for as long as possible usually overnight. Blocking buffer was removed and the membrane incubated for 1-2 hours in diluted primary antibody (1/500 to 1/1000) in TBS/1% gelatin with shaking. Unbound antibody was washed away with 4 X 15mls TBS, 0.2% Triton X-100 for 15 minutes each wash. Alkaline phosphatase-conjugated IgG was used as the secondary antibody at a concentration of 1/8000 (anti-rabbit) or 1/1000 (anti-mice) in TBS/0.1% gelatin, incubation was for 1-2 hours followed by 4 X 15mls washes with TBS, 0.1% Triton X-100 for 15 minutes each wash. Colour development of the

membrane was carried out in AP buffer (100mM Tris.HCl pH 9.5, 100mM NaCl, 50mM $MgCl_2$) with the addition of a 1:100 dilution of NBT (Nitroblue tetrazolium) @ 50mg/ml and 1:200 dilution of BCIP (5-bromo-4-chloro-3-indoyl phosphate) @ 100mg/ml. Once development of the membrane was compete the reaction was stopped by washing the membrane several times with 1% acetic acid and the membrane photographed while wet.

2.15.3 Detection of proteins

2.15.3.1 Coomassie blue.

The gel was incubated in 100ml of staining solution (10% isopropanol, 10% acetic acid, 0.1% Coomassie blue) at 65^0C for 1 hour and then destained for 2 hours at 70^0C in 200ml destain solution (5% methanol, 7.5% acetic acid).

2.15.3.2 Silver staining.

The gel was transferred to a solution containing 40% ethanol, 5% formaldehyde for 30 min and then rinsed in dH_20 for 30 minutes prior to incubation in 50% methanol overnight. Following incubation for 30 minutes in dH20 containing approximately 20mg DTT the gel was transferred to 200ml of 0.1% silver nitrate for 30 minutes and then developed in developer solution (18g sodium carbonate, 300μl 37% formaldehyde in 600ml dH_20. Colour development was fixed by the addition of 10g citric acid.

2.15.4 Preparation of acetone powders from bacterial cultures.

A 100ml culture of *E.coli* Y1090 in LB broth was pelleted by centrifugation at 6,000 rpm for 20 minutes. The pellet was resuspended in 4mls of 0.9% NaCl on ice for 5 minutes and mixed well with 8mls of acetone (-20^0C) per 2mls of cell suspension. This was incubated on ice for 30 minutes and the precipitate collected by centrifugation at 10,000g for 10 minutes. The pellet was resuspended in fresh acetone (-20^0C) and mixed vigorously on ice for 10 minutes. Following centrifugation for 10 minutes at 10,000g the pellet was spread onto a sheet of clean filter paper and allowed to air dry at room temperature. Once dry the powder was removed to an air tight container until used. When the powders were required they were added to the antiserum at a final concentration of 1% (w/v) and incubated for 30 minutes at 4^0C. The mix was spun at 10,000g and the supernatant used as the antibody source.

2.15.5 Bradford assay.

The protein concentration of cell extracts or purified expression products was determined by a Bradford assay using the Bio-Rad micro assay reagent (Bradford, 1976). The dye reagent

concentrate was diluted 1 in 5 with distilled water and 200μl of this was mixed with 100μl of sample, diluted if necessary. This was carried out in a microtitre plate (Falcon). The absorbance at 595nm was measured using a Titerek Multiscan plate reader. The protein concentrations were calculated from the absorbance readings using a calibration curve from known concentrations of BSA.

2.16 Whole cell extracts of *Coix* seeds and leaf tissue.

After harvesting, both leaf and seed tissue were stored at -80°C. The hulls of the fruiting bodies were removed, frozen immediately in liquid nitrogen and stored at -80°C until required. All of the following preperations was carried out in a 4°C cold room.

The plant tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle, transferred to 50ml polypropylene tubes containing chilled extraction buffer (10mM Tris pH 7.5, 1mM EDTA pH 8.0, 250mM KCL, 0.5mM DTT, 10% glycerol and 1mM PMSF dissolved in DMSO) and the mash homogenised using a polytron PCU (Kinematica) for 5 x 20 sec. bursts at 4°C. The homogenate was centrifuged at 15,000g for 20-30 minutes and the supernatant stored on ice for 15-30 minutes. The proteins were precipitated by slowly adding ammonium sulphate (0.4g/ml supernatent) to the supernatent and incubating the mix with stirring for 1 hour at 4°C. The precipitate was collected by centrifugation at 15,000g for 20 minutes and the resulting pellet resuspended in a minimal volume (usually 1-2 ml) of resuspension buffer (10mM Tris.HCl pH7.5, 1mM EDTA pH 8.0, 0.5mM DTT, 10% glycerol and 1mM PMSF). This was dialysed overnight in 2 litres of dialysis buffer (10mM Tris.HCl pH 7.5, 1mM EDTA pH8.0, 50mM KCl, 0.5mM DTT, 10% glycerol, 1mM PMSF). Following dialysis the protein solution was centrifuged in an Eppendorf at 12,000g for 10 minutes and the supernatant aliquoted into 100μl fractions and snap frozen in liquid nitrogen. Samples were frozen at -80°C until required.

2.17 Expression and purification of glutathione-S-transferase fusion proteins

2.17.1 Large scale purification of GST-fusion protein

A single colony of the pGEX3X (containing the insert) transformant was inoculated into 50ml LB/ampicillin/tetracycline medium and grown o/n at 37°C in shaking incubator. This culture was diluted 1:10 into 500ml fresh LB/ampicillin/tetracycline medium in a 1-litre flask, and grown 1 hour at 37°C. After this the fusion protein was induced by adding 100mM IPTG to 0.1mM and incubation continued for an additional 4 hours. The cells were collected by centrifugation for 10 minutes at 6000 rpm and the supernatent discarded. The pellet was resuspended in 6 ml ice cold PBS (150mM NaCl, 16mM NaH_2PO_4, 4mM Na_2HPO_4, pH7.3)

and transferred to a 30ml Corex tube. The cells were lysed using a probe sonicator with a 2mm diameter probe. The frequency and intensity of sonication was adjusted so that no frothing occured, normally this was 6 X 10s bursts at maximum output with 20s intervals between each sonication. Lysis was complete when the cloudy cell suspension becomes translucent and/or a change in colour occured from a rich straw brown to a dull grey brown (lysed cells). The cell debris was pelleted by centrifugation at 10000 rpm for 25 minutes. The supernatant which contains the soluble fusion protein was removed and loaded onto a glutathione-agarose column previously equilibrated with PBS/Triton X-100. The column was washed several times (approximately 4 column volumes) with PBS/TritonX-100 and finally with 1 column volume of PBS. The fusion protein was eluted by adding 10ml 50mM Tris-HCl (pH 8.0)/5 mM reduced glutathione to the column and collecting 500μl fractions of the elutant. 2μl of each fraction was analysed for protein concentration by carrying out Bradford assays. The fractions which contained protein were pooled into the one tube and an aliquot analysed by SDS-PAGE. Eluted protein aliquots were stored at -70°C.

2.17.2 Preparation of the glutathione-Sepharose column

Gluathione Sepharose 4B is an affinity chromatography gel designed for the purification of recombinant derivatives of glutathione-S-transferase expressed from the GST system The glutathione ligand is attached by coupling with the oxirane group of an epoxy-activated gel matrix, and is complementary to the glutathione binding site of glutathione-S-transferase. Elution of bound protein is accomplished using mild conditions (5mM glutathione) instead of the harsh acid washes required with some systems. These mild elutions help to preserve the antigenicity and functionality of the eluted protein.

2.18 Cleavage of GST-fusion protein with factor Xa.

Fusion proteins expressed using pGEX3X contain an amino acid sequence between the GST carrier and the foreign polypeptide that is cleaved by the site specific protease factor Xa, a blood coagulation factor (Nagai and Thorgerson, 1984). Obviously one must verify prior to cleavage (by inspection of the sequence) that the cloned polypeptide lacks sites recognised by factor Xa. Two alternatives are available for cleavage of fusion protein; to conduct the cleavage reaction on the pure fusion protein in solution (Smith and Johnson, 1988), or to cleave the foreign polypeptide from GST while it is still bound to glutathione-agarose beads (Gearing *et al.*, 1989).

2.18.1 GST-fusion protein cleavage on the column.

The normal procedure for the expresson and purification of the fusion peptide up to loading onto the glutathione-Sepharose column was carried out (section 2.17.1 and 2.17.2). The column was washed with 2 column volumes of each of the follwing buffers sequentially;

PBS-Triton X-100

PBS

50mM Tris pH 8.0

Wash buffer- 50mM Tris pH 7.5, 150mM NaCl

Cleavage buffer- Wash buffer/1mM CaCl

The cleavage buffer was allowed to drain until approximately 300μl remained on the surface of the Sepharose. The amount of fusion protein bound to the beads was estimated by Bradford assay (section 2.15.5) . To the remaining slurry, factor Xa was added (1% w/w fusion protein) and the column mixed well and incubated o/n at 25°C. The released peptide was recovered by washing the beads with 100μl aliquots of wash buffer ten times. The elutant was centrifuged through a centricon column for 1 hour at 4,000 rpm to separate the factor Xa from the 3kDa peptide fragment. The α-amylase-inhibitor should pass through the centricon filter into the collection vial, whereas the factor Xa remains on the other side of the filter. After elution of the foreign polypeptide the column was washed with 50mM TrisHCl pH 8.0, 5mM reduced glutathione, GST alone should be eluted. The eluted proteins in each fraction were analysed by SDS-PAGE and Shägger gel electrophoresis (Shägger *et al.*, 1987).

2.18.2 GST-fusion protein cleavage off the column

The GST-fusion protein was prepared as described in section 2.17 and eluted from the column in 2.5 ml of glutathione-TrisHCl and loaded onto a PD10 column to eliminate the glutathione prior to cleavage. The protein was eluted with 3.5ml of wash buffer in 500μl aliquots. 5μl of each aliquot was seperated by SDS-PAGE to check the approximate concentration of the protein. Peak fractions were pooled and the concentration of protein estimated at 1.7mg/ml. The sample was made 1.0mM CaCl and 0.01% SDS with 500mM and 20% stock solutions respectively. Factor Xa was added at a final concentration of 1%/gwt of protein, mixed well and cleavage allowed to occur at 25^{0}C for 1 hour. The mix was loaded onto a glutathione-Sepharose column which had been equilibrated with wash buffer. The protein was eluted with 6 X 500μl aliquots with wash buffer and 15μl of each sample analysed by SDS-PAGE and Shägger gel electrophoresis.

Finally the column was eluted with 6 X 500μl washes with 50mM TrisHCl pH 9.5/gluathione to elute the GST portion, 15µl of elutant from each aliquot was analysed by SDS-PAGE and Shägger gel electrophoresis.

2.19 Production of a λgt11 cDNA expression library

2.19.1 cDNA synthesis.

cDNA was prepared using a cDNA synthesis kit (Pharmacia) which uses a method adapted from Gubler and Hoffman (1983).

Approximately 5µg of poly A^+ RNA from seeds of *Coix lacryma jobi* was placed in a sterile Eppendorf tube and RNAase free water was used to bring the volume up to 20µl. This was heated to 65^{0}C for 10 minutes in order to destroy any secondary structures which may have formed. The solution was then placed on ice. The kit provides a tube labelled "first strand reaction mix" which contains the following components at concentrations optimised for reverse transcription :

(a) FPLC*pure* TM Cloned Moloney Murine Leukemia Virus (MMLV) Reverse transcriptase

(b) RNA guard, a commercial preparation of RNase inhibitor

(c) RNase/DNase free bovine serum albumin (BSA)

(d) Oligo $d(T)_{12-18}$ primer

(e) dATP, dCTP, dGTP, dTTP

To this tube was added 1µl of the supplied DTT solution. The contents were mixed by pippeting and incubated at 37^{0}C for 1 hour. During this stage the oligo $d(T)_{12-18}$ primer base pairs with the poly (A) residues to act as a primer for the MMLV reverse transcriptase which synthesises the first cDNA strand in a 5' to 3' direction using the mRNA as a template. Following incubation the entire contents of this tube were transferred to a tube labelled "second strand reaction mix" which contained the following components :

(a) *E.coli* RNase H

(b) *E.coli* DNA polymerase I

(c) dATP, dCTP, dGTP, dTTP

After mixing, incubation was at 12^{0}C for 1 hour followed by one hour at 22^{0}C. During these incubations the RNase H nicks the RNA strand of the cDNA : RNA hybrid formed in the first step and the DNA polymerase I uses nick translation to synthesise the 2nd strand.

Next 1μl of supplied Klenow fragment was added to the reaction which was incubated for a further 30 minutes at 37^{0}C. This stage ensures that the cDNA molecule will be blunt ended ready for the addition of adaptors. The reaction solution was then phenol/chloroform extracted and centrifuged for 1 minute at 13,000rpm. The upper aqueous phase was purified on Pharmacia" spun columns" which contained Sephracyl S-300 in distilled water with 0.15% Kathon CG (a preservative). These were preequlibrated in ligation buffer prior to spinning for 2 minutes in a bench cenrifuge at 3,000g to compact the Sephacryl S-300. After the DNA was applied the spin was repeated and the effluent collected in a Eppendorf. To the cDNA containing elutant, 5μl of the provided EcoR1/Not1 adaptor solution was added along with 1μl ATP solution and 3μl T4 ligase. After mixing the reaction was left at 12^{0}C overnight. During this time the adaptor molecules become ligated onto the blunt end of the cDNA molecules. The adaptor design is such that methylation is no longer required at this stage. The adaptors are composed of two non-self-complementary oligonucleotides which form a duplex with a phosphorylated blunt end and a non phosphorylated EcoRI overhang:

AATTCGCGGCCGC

GCGCCGGCGp

This ensures that only one adaptor can be ligated on to each cDNA terminus and the only other products are adaptor dimers rather than large multimers which can form with conventional linkers.

Following overnight incubation the sample was heated at 65^{0}C for 10 minutes and then incubated at 37^{0}C for 30 minutes during which the enzyme adds the terminal phosphate group to the EcoRI terminated cDNA ready for ligation into the EcoRI site of the bacteriophage vector. The solution was extracted with an equal volume of phenol/chloroform and the upper aqueous phase applied to a second spun column pre-equilibrated in STE buffer (10mM Tris-HCl pH7.5, 1mM EDTA, 150mM NaCl). This stage separated the cDNA from the excess and dimerised adaptors which remain on the column. Column elutant was collected in an Eppendorf tube.

2.19.2 Ligation into a bacteriophage vector.

The bacteriophage cloning vector λgt11 (Young and Davis, 1983) was used for library construction. This has a single EcoRI cloning site located in the *lac*Z gene 53 base pairs upstream from the translation termination codon. This vector can accomodate up to 7.2 Kb of foreign cDNA. It was chosen as it gives the flexibility of being able to either immunoscreen a cDNA library (Young and Davis, 1983), since foreign DNA sequences have the potential to be exprssed as β-galactosidase fusion proteins, or use conventional radiolabelled-nucleic acid probe techniques. λgt11 has a cI857 mutation which produces a temperature sensitive repressor protein inactive at 42^0C. In addition it has the amber mutation S_{am}100, which renders the phage lysis defective in host strains which do not contain the amber suppresor mutation *sup*F. λgt11 was supplied by Pharmacia as vector "arms", i.e. λgt11 DNA which had been restricted with EcoRI and then phosphatase treated to remove the terminal phosphate group on each arm. This is to prevent the two arms religating with each other during the construction of the library. However, phosphatasing is an inefficient process and so some parental phage are always formed and are present in the library. λgt11 arms are supplied at a concentration of 500μg/ml. The following mixes were set up for precipitation:

	A	B	C
Column elutant	6μl	3μl	2μl
STE buffer	to 30μl	to 30μl	to30μl
λgt11 arms	2μl	2μl	2μl
Total volume	32μl	32μl	32μl

To each of the three mixes was added 1μl 3M sodium acetate and 60μl ice-cold 95% ethanol. The tubes were placed at -70^0C for 15 minutes to coprecipitate the cDNA and vector. Pellets were collected by centrifugation for 10 minutes at 13,000rpm, vacuum dried and redissolved in 9μl sterile dH_2O and 1μl of the supplied ATP solution at a 1/10 dilution together with 1μl of the T4 DNA ligase provided. Solutions were mixed and left at 12^0C overnight such that cDNA molecules could ligate with the vector arms.

Following ligation 2μl aliquots were packaged *in vitro* using packaging extracts obtained from Promega. Dilutions of the paCkaging mix were plated out onto *E.coli* Y1088 (Young and Davis, 1983) to titre the number of infective phage. The number of recombinant phage in the library was calculated by plating a countable number of plaques onto a lawn of Y1088 cells using 3ml of Top agar containing 40μl of 40mg/ml Xgal and 40μl of 1M IPTG and

incubating overnight at 42^{0}C. Parental phage produce blue plaques on the *sup*F *lac*I^{Q} containing host Y1088 in the presence of the *lac* operon inducer IPTG and the chromogenic β-galactosidase substrate Xgal, whilst recombinant phage produce clear plaques.

2.19.3 Preparation of cells for bacteriophage infection.

E.coli Y1088 and Y1090 were used for the propagation of λgt11 phage. Both strains were prepared for infection in the same manner. A single colony of each strain was inoculated from an LB agar plate into 5mls of LB broth and incubated at 37^{0}C overnight. 1ml of this culture was used to inoculate 100mls of LB broth supplemented with maltose (final concentration 0.2%) and this incubated overnight at 37^{0}C with shaking. This provided stationary phase cultures which were divided equally over 4 sterile falcon tubes. Cells were collected by centrifugation for 10 minutes at 3,000g in a Wifuge lab 500E bench centrifuge. Supernatants were discarded and the cells resuspended in a total volume of 10ml of SM buffer. Cells were stored at 4^{0}C for up to a week.

2.19.4 Amplification of a cDNA library.

The cDNA library was amplified in *E.coli* Y1088 which is *hsd* R^{-} *hsd*M^{+}. It is important that poorly growing recombinants are not selected against during library amplification. Such poorly growing phage can arise if toxic polypeptides are produced in the form of the β-galactosidase fusion proteins. Therefore *E.coli* Y1088 is ideal since it produces excess *lac* repressor so inhibiting the production of any fusion proteins until derepressed with the inducer IPTG when required.

After calculating the size of the library and percentage recombinants the whole library was plated onto *E.coli* Y1088 over a series of LB agar plates in the normal manner. After incubation overnight at 42^{0}C the top agar containing the plaques was scraped into 50ml sterile polypropylene tubes containing 30ml SM buffer. The tubes were vortexed well and the phage allowed to diffuse out of the top agar overnight at 4^{0}C. Debris was pelleted by centrifugation at 3,500rpm for 15 minutes in a bench centrifuge. The supernatent was transferred to fresh tubes and PEG-8000 was added to 10%. Phage were precipitated overnight at 4^{0}C and collected by centrifugation as before. The pellet was resuspended in 15ml of SM buffer and extracted with equal volume of chloroform by spinning at 10,000 rpm at 4^{0}C. Extraction was continued until the interface was clean and the phage solution was stored in 0.5ml aliquots in eppendorfs at 4^{0}C. A drop of chloroform was added to each tube to prevent bacterial contamination. The amplified library was titrated as described in section 2.20.1.

2.20 Immunoscreening of a λgt11 expression library.

2.20.1 Library titration.

It is important to determine the phage titre of the library to be screened in order to screen a representative amount of plaques from the library. The optimal plaque forming units (pfu) required for screening is typically 2-3 fold higher than that required to produce a dense field of individual plaques without total lysis of the bacterial lawn.

To titrate the phage :

1. Serial dilutions of the packaging mixes in SM buffer were made.

2. 10μl of each dilution was plated out on *E.coli* Y1088 and incubated at 37°C overnight.

3. The plaques were counted to determine the number of pfu/ml

2.20.2 Plating phage for screening.

2-3 day old plates (150mm petri dishes), which had been incubated at 37°C for 1 hour were used. Approximately 5 x 10^4 pfu were adsorbed with 600μl of Y1090 cells for 15 minutes at 37°C and then combined with 7mls of 0.8% top agarose. This mix was poured onto prewarmed plates and allowed to solidify at room temperature. The plates were incubated at 42°C until small plaques were visible (approximately 3.5 hours). Nitrocellulose filters which had been incubated in 10mM IPTG for 30 minutes and then air dried were placed onto the surface of the top agar taking care to exclude air bubbles. The plate now with filter, was incubated again at 37°C for 3.5 hours. The orientation of the nitrocellulose filter in relation to the plate was marked by piercing the filters in several places. The plaques were lifted by carefully removing the nitrocellulose filters from the plates. A duplicate filter was prepared by applying a second IPTG-nitrocellulose filter after removal of the first and returning the plate to 37°C for 4 hours or more.

The filters were washed in TBST for 15 minutes to remove any top agarose and then washed a further 3-5 times with 10mls per filter of TBST for 15 minutes. The filters were immersed in blocking solution (TBS/1% gelatin) and blocked overnight at room temperature in order to block remaining protein sites.

The filters were transferred to 10ml of fresh blocking solution containing a 1/500 dilution of the primary antibody, which had been treated with acetone powders, and incubated at room temperature with shaking for 3 hours.

The filters were washed 5 times in 10ml of TBST for 5 minutes each time to remove any unbound or non specifically bound antibody. The filters were transferred to fresh 10ml of blocking solution containing a 1/8,000 dilution of anti-rabbit alkaline phosphatase conjugate and incubated with shaking for 3 hours at room temperature. The filters were washed 5 times in 10 ml of TBST and a final wash in TBS.

The filters were transferred to 20 ml of colour development solution (100mM Tris/HCl pH 9.5, 100mM NaCl, 5mM $MgCl_2$) containing NBT and BCIP at final concentrations 0.3mg/ml and 0.15mg/ml respectively. The reaction was allowed to proceed in the dark until positive reactions were clearly visible. The colour development was terminated by washing the filter in 1% acetic acid several times.

2.20.3 Purification of positive plaques.

After a primary screen, positive plaque were identified by aligning the signals on the nitrocellulose filters with the original agar plates. Positive plaques were removed with the wide end of a pasteur pipette and placed into 1ml of SM buffer. Due to the plaque density on the original plate it was never possible to remove a single plaque without taking other non positive plaques as well. This meant that a second round of screening had to be performed on dilutions of the phage plug containing the positive signal so that an individual positive plaque could be isolated.

The SM buffer containing the agar plug was vortexed and left for at least 1 hour to allow the phage to diffuse out. Dilutions of the phage suspension were plated onto *E.coli* Y1090 cells as before and plates were selected which had well seperated plaques. These were rescreened in the same manner as the first round plates and positive plaques stored in SM buffer at $4^{0}C$ with a drop of chloroform added to prevent bacterial growth.

2.21 Oligonucleotide screening of the λgt11 cDNA library

Phage were plated out for screening as described in section 2.20.2, all buffers used are as described in section 2.10.15.2.

Nylon Hybond-N was cut into squares slightly smaller than square diagnostic plates. These were placed onto the surface of the top agar taking care to exclude air bubbles. Alignment was by several holes made with a sterile hyperdermic needle, the position of which was marked on the base of the plate with a felt pen. Filters were overlayed onto plates for 2 minutes and then transferred to sheets of 3MM paper which had been soaked in denaturing buffer. The filters were placed so that plaques were facing upwards and left for 5 minutes

before transferring to 3MM paper soaked in neutralising buffer for a further 5 minutes, then finally to 3MM paper soaked in wash solution for 5 minutes. DNA from the phage plaques was bound to the nylon by exposure to U.V. radiation for 3 minutes.

Filters were prehybridised for at least 2 hours in prehybridisation buffer in a Techne hybridisation oven at 50^{0}C. The buffer was removed and replaced by 10ml of hybridisation buffer containing the single stranded oligonucleotide probe. Hybridisation was for approximately 16 hours at 50^{0}C. Washing was in 6 X SSC, 0.1%SDS (at room temperature or 4^{0}C) for 4 times with 5 minute incubations. After the final wash filters were exposed to X-Ray film as described in section 2.11 for up to a week.

2.22 Preparation of phage DNA.

2.22.1 Plate lysate method

The method used was an adaptation of the plate lysate method in Sambrook *et al.* (1989).

All steps took place in Eppendorf tubes and centrifugation was always in the microcentrifuge at 13,000rpm. Single positive phage plaques were removed from agar plates with the fat end of a pasteur pipette and placed in 1ml of SM buffer. These were vortexed vigourously and left for at least 2 hours to allow the phage to diffuse out from the agar plug. 400μl of this phage suspension was added to 50μl of *E.coli* Y1088 plating cells and left at room temperature for 15 minutes to infect before being added to 3ml of Top agar and poured over a dry pre-warmed LB plate. Once the top agarose had set the plates were incubated at 37^{0}C overnight until confluent lysis was visible. 4ml of SM buffer was added to each plate and rotated slowly on a rocking platform for at least 2 hours. 1ml aliquots of the SM suspension was transferred into eppendorfs and spun for 5 minutes to pellet any bacterial debris. Supernatents were placed in fresh eppendorfs and made 10% PEG-8000 and 4% NaCl from 40% stock solutions. Tubes were incubated on ice for 1 hour to precipitate the phage then spun for 15 minutes to collect the phage pellet. Supernatents were discarded and pellets were resuspended in 200μl SM buffer. Solutions were then made 200μg/ml with RNAase and DNAase then incubated at 37^{0}C for 20 minutes to destroy bacterial RNA. Solutions were extracted with an equal volume of chloroform until the interface was clean. The solutions were then extracted once with phenol / chloroform and once with chloroform / isoamyl alcohol. Phage DNA was precipitated with by the addition of 1/10 volume 3M sodium acetate and 2 volumes of ice cold 95% ethanol. If phage DNA could not be seen immediately the tubes were placed at -20^{0}C for 30 minutes. DNA was collected by centrifugation for 10 minutes, washed with 70% ethanol, vacuum dried and resuspended in 50-100μl of sterile distilled water depending on the size of the pellet.

2.22.2 Liquid culture method.

This method is essentially as described in Sambrook *et al.* (1989). Using a pasteur pipette a single, well-isolated bacteriophage plaque was picked and incubated for 4 hours in 1ml of SM buffer containing a small drop of chloroform. 500μl of this suspension was added to 500μl of *E.coli* Y1088 plating cells and incubated at room temperature for 2 hours. This suspension was used to inoculate 50ml of L-broth containing 0.5ml 500mM CaCl and 50μl 50mg/ml ampicillin and the culture incubated at 42^{0}C with agitation, until lysis occured (normally 5-7 hours). Lysis was determined visually, the culture should become clear with very little debris visible. 2.5ml of chloroform was added to the culture and incubated with agitation for for a further 10 minutes at 42^{0}C. The lysate was transferred to 2 X 50ml polypropylene tubes and centrifuged at 3,000rpm for 10 minutes at room temperature. The supernatent was transferred to a fresh centrifuge tube and RNAase and DNAase treated at final concentrations of 10μg/ml for 15 minutes at 37^{0}C. An equal volume of a solution containing 20% w/v PEG-8000 and 2M NaCl in SM buffer was added to the supernatent and incubated for 1 hour in ice water. The precipitated phage particles were recovered by centrifuation at 12,000rpm for 10 minutes at 4^{0}C and the supernatent removed by gentle aspiration. The phage paricles were resuspended in 1.5ml of TE (pH 8.0), and resuspended by gentle vortexing. After the addition of 15μl of 10% SDS the solution was incubated for 5 minutes at 68^{0}C followed by the addition of 30μl of 5M NaCl. The bacteriophage were purified by extracting once with phenol:chloroform and once with chloroform alone, transferring the aqueous phase to a fresh tube between extractions. An equal volume of isopropanol was added to the aqueous phase and the mixture stored at -70^{0}C for 15 minutes followed by recovery of the DNA by centrifugaton at 12,000rpm for 15 minutes. The pellet was washed with 70% ethanol, vacuum dried and resuspended in 50μl of TE (pH 8.0).

2.23 Immunisation schedule for the production of polyclonal antibodies.

The basic method is outlined in Harlow and Lane (1988).

A large scale preparation of the fusion peptide was carried as in section 2.17. 100μg of GST-fusion peptide in 100μl of 50mM TrisHCl pH 9.5 was mixed by vortexing with 100μl of complete Freund's adjuvant. This was injected into 5 female mice intraperitoneally. After 14 days the mice were boosted with half the amount of protein but using incomplete Freunds. Tail bleeds were collected from each mouse 10 days after the boost and the titre of antibody checked by Western blotting. Two more boosts were carried out before the terminal bleeds were carried out. A non-immunized mouse was terminally bled to provide non-immune serum as a control.

The bleeds were incubated at 37^{0}C for 1 hour with shaking and then incubated at 4^{0}C overnight for clotting to occur. The serum was recovered following centrifugation for 10 minutes at room temperature.

2.24 Affinity purification of polyclonal antibodies.

This was based from the method of Robinson *et al.* (1988) and is detailed below.

0.5 mg of GST-fusion peptide in SDS sample buffer was applied to the total width of an SDS vertical slab gel and the protein seperated by electrophoresis at 150 V constant current until the bromophenol blue reached the bottom of the gel (section 2.15.1). Protein was transferred to nitrocellulose using a Biorad Western blotter (section 2.15.2) and transfer checked by staining with Ponceau-S. The strip of nitrocellulose containing the antigen was cut away from the rest of the filter. The strip was blocked by incubation in blocking solution overnight and then transferred to 40ml fresh blocking solution containing a 1/500 dilution of antibody and incubated overnight at room temperature with shaking. The antibody bound to the antigen was eluted by 2 consecutive incubations in 0.1 M glycine/HCl buffer, pH 2.5. The first for 5 minutes and the second for 10 minutes. The elutant was collected and then immediately diluted five fold with blocking solution. They were then dialysed against three changes of 1000 vols. of TBS over 24 hours and stored at 4^{0}C. The efficiency of purification was analysed by probing Western blots of crude protein extracts of seeds and the GST-fusion peptide with the affinity purified antibody..

The presence of the antigen-antibody complexes were detected by using ^{125}I-labelled goat anti-rabbit immunoglobins (Sigma). In brief, following incubation with primary mice antibody, filters were transferred to rabbit anti-mice conjugated secondary antibody followed by incubation in in ^{125}I-labelled antibody and autoradiography.

2.25 Amplification of cDNA and genomic DNA using the polymerase chain reaction

Template genomic DNA was prepared by the method oulined in section 2.12.

Template cDNA was prepared using the first strand reaction mix from the cDNA synthesis kit as outlined in section 2.19.1

All solutions and tips were as sterile as possible to aviod contamination by foreign template DNA's. Prior to use Gilson pipette ends were washed thoroughly with water and detergent.

The following reagents were mixed on ice in sterile 1.5ml Eppendorf tubes;

Sterile distilled water		Xμl
10 x reaction buffer		10μl
Stock dNTP's	16μl	(1.25 mM of each)
Template DNA		0.5-1.0 μg
Primers 5' and 3'		Final concentration 1uM
Total volume		99μl

(Commercial dNTP's from Boheringer at a concentration of 100mM were used as stock solutions. 12.5μl of each dNTP stock were mixed and added to 950μl of sterile dH_2O).

A control reaction was set up in which no tempate DNA was added to monitor contamination by foreign DNA's.

The reagents were mixed well and imediately prior to use, 1μl of Taq polymerase (Promega) was added. All reaction mixtures were covered with 200μl of mineral oil.

The tubes were coated in vacuum grease to increase thermal conductivity, placed in the thermal block and the appropriate program selected. Programs varied according to the annealing temperature but the basic programme was as follows;

1.

Melting	92^{0}C	1.5 minutes
Annealing	45-65^{0}C	1.5 minutes
Extension	72^{0}C	3.0 minutes

FOR 30 CYCLES

This programme immediately followed programme 1.

2.

Melting	92^{0}C	1.5 minutes
Annealing	45-65^{0}C	1.5 minutes

Extension	72^{0}C	5 minutes
	FOR 1 CYCLE	

After the 31 cycles were completed the tubes were removed from the heating block and briefly centrifuged to spin down the condensate. An aliquot of the sample (usually 20µl) was analysed by agarose gel electrophoresis.

2.26 Construction of T-vectors.

This method was described by Marchuk *et al.* (1990).

Bluescript plasmid was digested with EcoRV and incubated with Taq Polymerase (1 unit/µg plasmid/20µl volume) using standard buffer conditions in the presence of 2mM dTTP for 2 hours at 70^{0}C. The absence of any other nucleotides in the reaction results in the addition of a single thymidine at the 3' end of each fragment. After phenol/chloroform extraction and ethanol precipitation the vector was ready for cloning.

2.27 DNA sequencing

2.27.1 Manual DNA sequencing

DNA sequencing was carried out by the dideoxynucleotide chain termination method of Sanger *et al.* (1977), using a United States Biochemical Sequenase 2 sequencing kit.

2.27.1.1 Preparation of template DNA.

Plasmid DNA was prepared from 50ml overnight culture using a Qiagen plasmid mini preparation kit.

2.27.1.2 Annealing template and primer

A sequencing primer, either pUC19 -40, or the forward and reverse primer for pGEX3X (gift of Dr. Cluness, University of Durham, Durham) was annealed to the template by mixing together 1µl of primer, 2µl reaction buffer with 7µl of DNA (approximately 3-5µg) and heating the mix at 65^{0}C for 2 minutes. The mixture was cooled to below 30^{0}C at room temperature (usually over a period of 1 hour) and then placed on ice.

2.27.1.3 Labelling reaction

To the annealed template-primer the following were added;

Template-primer (above)	10.0μl
DTT 0.1M	1.0μl
Diluted labelling mix	2.0μl
[α-^{35}S] dATP	0.5μl/5μCi
Sequenase Version 2.0 DNA Polymerase	2.0μl

This was mixed well and incubated for 2 minutes at room temperature for the labelling reaction to occur.

2.27.1.4 Termination reaction

4 Eppendorf tubes were labelled G, A, T and C and were filled with 2.5μl from the ddGTP, ddATP, ddTTP and ddCCP termination mixes. These were prewarmed to 37^{0}C and to each tube was added 3.5μl from the labelling reaction. Incubation at 37^{0}C was continued for 5 minutes, followed by termination with addition of 4μl of stop solution. Samples were stored on ice until required for gel electrophoresis.

2.27.1.5 Denaturing gel electrophoresis

The gel apparatus used was the Biorad Sequi-Gen^{TM} sequencing gel this was assembled following manufacturers instructions.

Gels were prepared approximately 3 hours prior to use and prerun at 2,000 volts until the running temperature of 50-55^{0}C was reached. A 5% acrylamide sequencing gel was made by mixing the following at 65^{0}C until the urea has dissolved:

42g urea

10ml 10 X TBE buffer

40ml dd H_2O

The mix was cooled slightly and then added to 12.5 ml of a 40% acrylamide stock solution. Polymerisation occured following the addition of 140μl 25% ammonium persulphate and 100 TEMED.

When the gel was ready for loading, the 4 samples were heated to 75^{0}C for 2 minutes and 4μl of the sample loaded immediately onto the gel in the order G, A, T, C. Electrophoresis

occured at 2,000 volts until the bromophenol blue reached the bottom of the gel. Electrophoresis was stopped briefly to allow loading of the 4 samples again as above in the adjacent lanes to the previous 4 loadings. Electrophoresis of the samples was finally stopped when the bromophenol blue from the second loadings had reached the bottom of the gel.

The gel was transferred to Whatmann 3MM filter paper and dried at 80^0C for 2 hours in a Biorad Model 583 Gel Dryer. Exposure was as described section 2.11 for 2 days at room temperature with direct contact between the gel and the film.

2.27.2 Automated sequencing

This was carried out using the Applied Biosystems 373A DNA sequencer.

2.28 Computer programs

DNA sequences were analysed by the DNA Strider programme. Sequence alignments were carried out using the Gap algorithms in the UWGCG suite of programmes at SERC SEQNET Daresbury. The EMBL data base searches were carried out using the OWL package on SEQNET at the SERC Daresbury facility.

2.29 Oligonucleotide synthesis.

Oligonucleotides were synthesised using an Applied Biosystems 381A DNA synthesiser according to manufacturers instructions.

CHAPTER 3

IMMUNOSCREENING A *COIX* SEED cDNA λGT11 EXPRESSION LIBRARY.

3.1 Introduction

3.1.1 cDNA library construction

Until 1983, almost all cDNA cloning was carried out using plasmid vectors, this was extremely laborious and often generated very unsatisfactory results. With the development of more efficient ways to synthesise cDNA (Gubler and Hoffman, 1983) and with the advancement of linkers, adaptors, methylases and packaging mixes a new set of bacteriophage vectors were developed, namely the λgt vectors , λORF8 and more recently λZAP.

The type of vector system employed when attempting to isolate cDNA clones is in part determined by the probe which will be utilised to screen for the clone of interest. Basically, there are 2 methods available for probe screening namely nucleic acid detection and immunological detection. As an antibody raised against the α-amylase inhibitor protein was available for screening, the latter method was employed using a λgt11 expression library (Young and Davis 1983, 1985). λgt11 is an expression vector that is used to construct libraries that are to be screened with immunological probes to isolate DNA sequences that code for specific antigens. λgt11 carries a copy of the *E.coli lacZ* gene, with a single Eco R1 cleavage site located 53bp upstream of the termination codon of the *lacZ* gene. Approximately 7.2 kb of foreign DNA can be cloned into this site. Coding sequences inserted in the correct open reading frame and orientation will be expressed to yield fusion proteins whose amino-termini consist of β-galactosidase sequences and whose carboxyl-termini consist of foreign polypeptides. Some of these fusion proteins will display antigenic epitopes that can be detected by their ability to react with specific antibodies and thus the cDNA clone of interest can be isolated.

The *lon* protease deficient host Y1090 is used to plate out λgt11 libraries for screening with antibody probes. *lon* protease deficiency increases the stability of the β-galactosidase fusion proteins without affecting cell growth. Y1090 is not defective for host-controlled restriction and modification enzyme activities. Therefore the initial packaging mix of libraries cloned in λgt11 should not be plated directly on Y1090 for screening, but should first be amplified through Y1088, which is *hsd*R^- *hsd*M^+. In addition to this to avoid creating a bias against relatively poorly-growing recombinants during amplification of the library, it is important to repress the production of toxic polypeptides (in the form of toxic β-galactosidase fusion

proteins) encoded by the recombinant phage. Therefore, *E.coli* Y1088, which produces the lac repressor, is used as the host to amplify the library.

3.1.2 Antibody probes

Immunological methods, can in principle, be used to detect any protein for which a specific antibody is available. The specificity of the antibody-antigen binding is defined by a small region (epitope) of the antigen. Some epitopes are extremely complicated topological structures formed by the folding of noncontiguous regions on one or more polypeptide chains whereas others are formed from stretches of adjacent amino acids on the same polypeptide chain (Harlow and Lane, 1988). Formation of these epitopes is dependant on the folding of the protein in *E.coli*, although localised epitopes may be formed in malfolded or non-functional proteins. The formation of the appropriate epitopes for antibody recognition is also dependant on the lengths of the cDNAs cloned with respect to the size of the protein of interest. If full length cDNAs are not cloned it is possible for fragments of protein to form simple epitopes and retain at least part of their immunological properties.

3.1.3 Available detection methods for visualisation of positive signals

Several antibody screening detection methods are available employing both radioactive ^{125}I-labelled anti-immunoglobulin and anti-immunoglobin-enzyme conjugates to detect the primary antibody antigen complexes. Horseradish peroxidase (HRP) and alkaline phosphatase (AP) are the available enzyme-linked detection systems. Alkaline phosphatase-conjugated secondary antibody was choosen in this study for several reasons. Dao (1985) suggested that AP-conjugates were capable of detecting antigens over an order of magnitude greater than the equivalent HRP-conjugate. Mierendorf *et al.* (1987) compared the relative sensitivities of the 2 systems. AP could detect antigen to a limit of 20-50pg, whilst the HRP conjugate failed to detect antigen of less than 200pg, following a library screen assay. On this basis it was decided to use alkaline phosphatase as the secondary antibody to screen the λgt11 expression library.

3.2 Characterization of antibodies for immunological screening

Polyclonal antibodies have been used succesfully to isolate genes using λgt11 cDNA expression libraries from several genomes. The nature of the antibody is essential for success. Ideally, polyclonal antibodies, which are absolutely specific for conformation independent epitopes that are present on both the native and denatured proteins and of high titre should be utilised. Realistically, it is reasonable to assume that antibodies which

produce specific, good signals on Western blots of the protein of interest will produce good signals in the λgt11 screening procedure (Harlow and Lane, 1988).

Two antibodies were available for the isolation of the α-amylase inhibitor cDNA, both had been raised in rabbit. Firstly, immunoserum raised against the *Coix* α-amylase inhibitor protein, and secondly immunoserum raised against a wheat germ endochitinase. Both were gifts from M.Ary, Rothamstead Experimental Station.

3.2.1 Description of antiserum raised against an α-amylase inhibitor protein.

The α-amylase inhibitor protein (Ary *et al.,* 1989) had been purified from seeds of *Coix* (mid-late stages of maturation) and used to raise polyclonal antiserum against the protein in rabbit (M.Ary, Rothamstead Experimental Station). She had characterised the antisera by probing the pure α-amylase inhibitor protein and by immunprecipitation of *in vitro* translation products synthesised by translation of poly A^+ RNA from seeds of *Coix.*

SDS-PAGE analysis of the α-amylase inhibitor protein had resolved a band of Mr 52,500Da under non-reducing conditions, which was replaced by a band of Mr of 26,400Da in the prescence of 2-mercaptoethanol. M.Ary (personal communication) had detected an immunopositive protein of approximately 26,000Da following Western blotting of the α-amylase inhibitor with this antisera.

M.Ary had also shown that *in vitro* translational products synthesised from polyA^+ RNA from *Coix* seeds (the same polyA^+RNA source was used for cDNA library constuction), and their subsequent immunoprecipitation with the α-amylase inhibitor antibody, had precipitated a polypeptide of the predicted molecular weight.

3.2.2 Description of antiserum raised against a wheat germ endochitinase

Although initially characterised as an inhibitor of insect α-amylase, the partial amino acid sequence of the *Coix* protein showed strong homology with those of endochitinases from various sources including barley seeds, and leaves of tobacco, potato and beans (Ary *et al.,* 1989). The authors also demonstrated endochitinase activity of the protein following the release of radioactivity from [^{3}H]chitin. Endochitinases are widespread in plants and are produced in a number of tissues such as oat leaves (Fink *et al.,* 1988), potato tubers (Gaynor, 1988), seeds of barley (Leah *et al.,* 1987) and wheat (Molano *et al.,* 1979). Endochitinases are also produced in response to damage or invasion by pathogens (in leaves of tobacco, bean, potato and maize). They have molecular weight values estimated by SDS-PAGE of between 25,000Da and 30,000Da and can be classified into subgroups with low and high pI

values called 'acidic' and 'basic' endochitinases respectively. The *Coix* protein is of the basic type.

Antiserum raised in rabbit, against an endochitinase from wheat (Molano *et al.*, 1979), was obtained from M.Ary. She had characterised the antibody as crossreacting with both the isolated α-amylase inhibitor protein and following immunoprecipitation, with the same *in vitro* translation product which the α-amylase inhibitor antiserum had precipitated.

3.3 Isolation and characterization of polyA+ RNA from the seeds of *Coix lacrhyma-jobi.*

PolyA+ RNA isolated from seeds in their middle to late stages of development (12-24 days after flowering) was a gift of M. Ary (Rothamstead Experimental station). The success of the cDNA library construction is directly dependant on the integrity of the mRNA before it is used as a template for cDNA synthesis. The following experiments were carried out to asses the suitability of the polyA+ RNA for cDNA synthesis.

3.3.1 The ability of the mRNA to direct the synthesis of high molecular weight proteins.

Approximately 10μg of *Coix* polyA+ RNA was used in an *in vitro* translation experiment using an Amersham rabbit reticulocyte *in vitro* translation kit (section 2.14.10). The translation products were labelled using ICN Radiochemicals Trans ^{35}S-Label containing 70% L-methionine [^{35}S] + 30% L-cysteine [^{35}S]. This allows all the methionine residues present in *in vitro* translation products to be labelled. Once the *in vitro* translation products were synthesised a 17μl aliquot was analysed by SDS-PAGE using a 12% running and 6% stacking gel (section 2.15.1). The gel was exposed to X-ray film and the film developed following storage for 4 days at -80^{0}C (section 2.11). The autoradiograph is displayed in Figure 3.1, lanes 1 and 5.

A wide range of polypeptides were synthesised the upper limit being approximately 106,000Da which suggested that the polyA+ RNA was intact enough to encode for these high molecular weight polypeptides. Figure 3.1, lane 3 shows *in vitro* translation of RNA's endogenous to the rabbit reticulocyte.

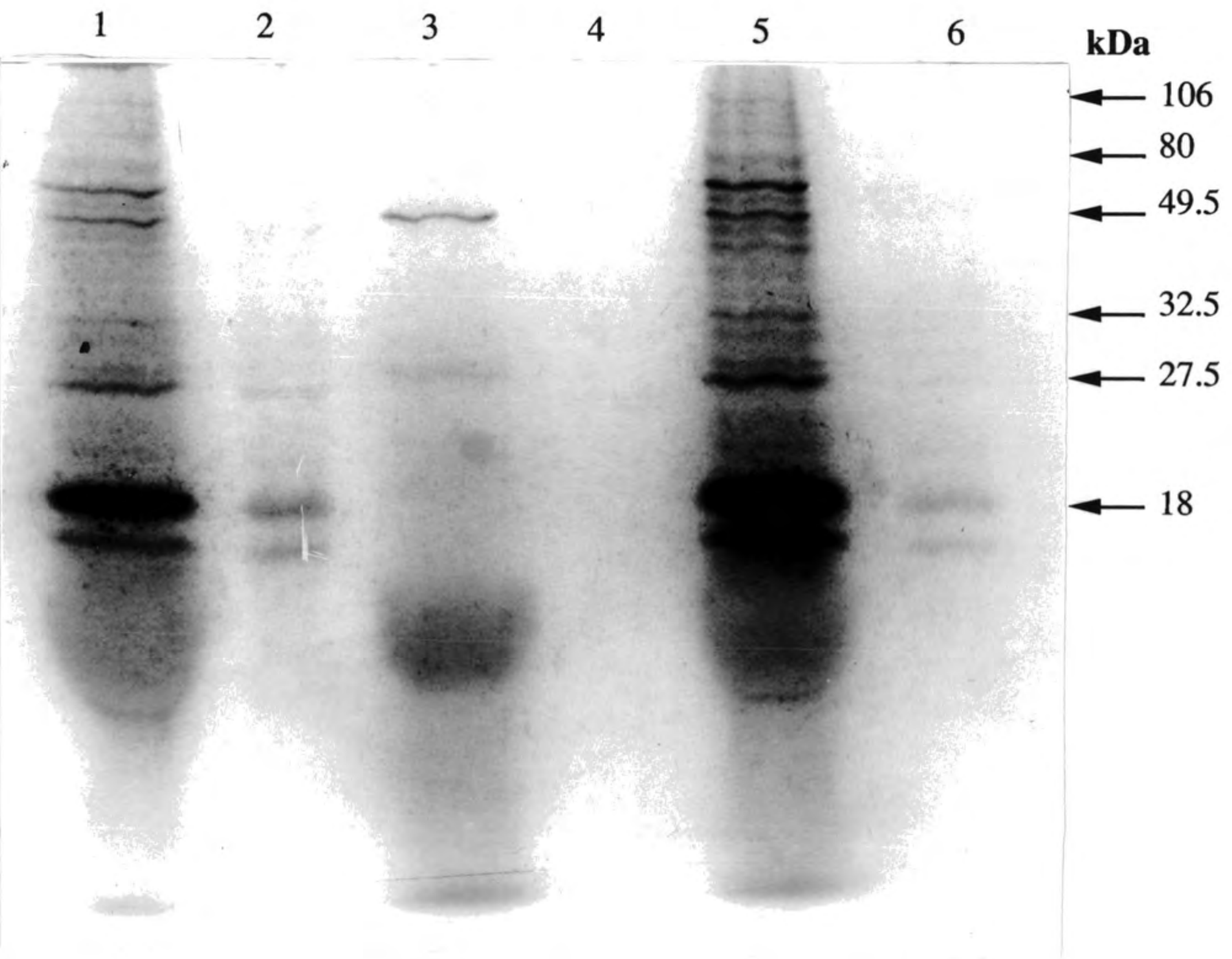
3.3.2 The ability of the mRNA to direct the synthesis of the α-amylase inhibitor.

It was crucial to determine whether the α-amylase inhibitor message was present in the polyA+ RNA template prior to cDNA synthesis and subsequent library constuction. M. Ary had previously established that the message encoding the α-amylase inhibitor polypeptide was present in the polyA+ RNA from this stage of *Coix* seeds. This was established by

Figure 3.1 ***In vitro*** **translation of polyA$^+$ RNA from seeds of** ***Coix*** **and subsequent immunoprecipitation of these products with α-amylase inhibitor immunoserum.**

10μg of *Coix* polyA$^+$ RNA was used in an *in vitro* translation experiment using an Amersham rabbit reticulocyte *in vitro* translation kit (section 2.14.1).
Translation products were immunoprecipiated with α-amylase inhibitor immunoserum (section 2.14.2) and the products analysed by SDS-PAGE, using a 12% runnung and 6% stacking gel (section 2.15.1).
The gel was exposed to X-ray film and the film developed following storage for 4 days at -80^{0}C (section 2.11).

Lane 1	*In vitro* translation of *Coix* seed polyA$^+$ RNA
Lane 2	Immunoprecipitation of translation products with a 1/5 dilution of α-amylase inhibitor immunoserum
Lane 3	*In vitro* translation reaction control; no *Coix* RNA
Lane 4	Immunoprecipitation control; no *Coix* RNA *in vitro* translation products immunoprecipitated with 1/50 dilution of α-amylase inhibitor immunoserum
Lane 5	*In vitro* translation of *Coix* seed polyA$^+$ RNA
Lane 6	*In vitro* translation of seed polyA$^+$ RNA; immunoprecipitation of products with a 1/50 diltion of α-amylase inhibitor immunoserum


1
2
3
4
5
6
kDa
106
80
49.5
32.5
27.5
18

translation of the mRNA *in vitro*, followed by immunoprecipitation of the translation products with anti-serum raised against the α−amylase inhibitor protein. Identification of the α-amylase inhibitor product from the total *in vitro* translation products was carried out. However, it was decided to confirm the results of these experiments again in Durham.

PolyA$^+$ RNA was translated *in vitro* and 37μl aliquots of the translation products incubated with 1/5, 1/50 and 1/500 dilutions of α-amylase-inhibitor anti-serum overnight at room temperature. The antigen-antibody complex was precipited by incubation with protein A-Sepharose (section 2.14.2) and the immunoprecipitation products analysed by SDS-PAGE (section 2.15.1). Aliquots of *in vitro* translation products were loaded alongside immunoprecipitation products for comparison as illustrated in Figure 3.1 lanes 2 and 6 and Figure 3.2 lane 3.

Lanes 3 and 4 in Figure 3.1 show that a 1/50 dilution of α-amylase inhibitor antibodies does not crossreact with any translation products endogenous to the rabbit reticulocyte.

Three translation products were immunoprecipitated with 1/5 and 1/50 dilutions of α-amylase inhibitor antiserum with molecular weights of approximately 17,000, 18,000 and 27,000Da, as estimated by SDS-PAGE. Only the 17,000Da protein was immunoprecipitated with a 1/500 dilution of the α-amylase inhibitor anti serum.

3.3.3 The ability of the polyA$^+$ RNA to direct the synthesis of long molecules of first strand cDNA.

A pilot first strand cDNA synthesis reaction was set up using approximately 0.5μg of poly A$^+$ RNA (section 2.19.1). The efficiency and yield of first strand synthesis was monitored by incorporating 50μCi of ^{32}P-dCTP into the reaction. A 5ul sample was counted in a scintillation counter, this gave a reading of 5549cpm/μl. 36μl (2 x 10^5 cpm) was analysed following electrophoreses in a 0.8% agarose/TAE gel containing ethidium bromide at 100v for 4 hours (section 2.10.10). 10.4μl (2 x 10^5cpm) of unincorporated nucleotides was also electrophoresed along with λPst 1 DNA markers. Following elecrophoresis the gel was photographed under ultra-violet light, wrapped in sellophane and autoradiographed overnight at -80^{0}C (section 2.11). The majority of cDNA labelled lies between 700b to 2kb, with the upper limit of the smear being approximately 3kb (Figure 3.3).

3.4 Construction of a λgt11 cDNA expression library.

The methods involved in cDNA synthesis and ligation into λgt11 vector are described extensively in 2.19.

Figure 3.2 ***In vitro*** **translation of** ***Coix*** **seed polyA$^+$ RNA**

Lane 1	*In vitro* translation of *Coix* seed polyA$^+$RNA
Lane 2	*In vitro* translation of *Coix* seed polyA$^+$RNA
Lane 3	*In vitro* translation of *Coix* seed polyA$^+$ RNA; immunoprecipitation of products with a 1/500 diltion of α-amylase inhibitor immunoserum

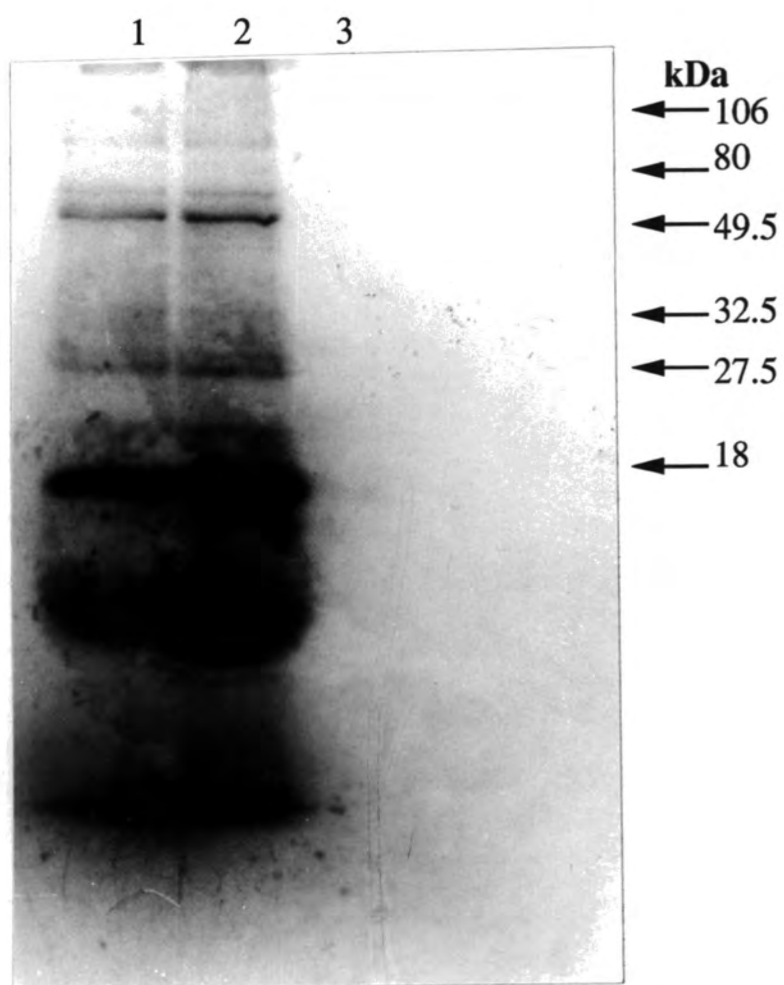
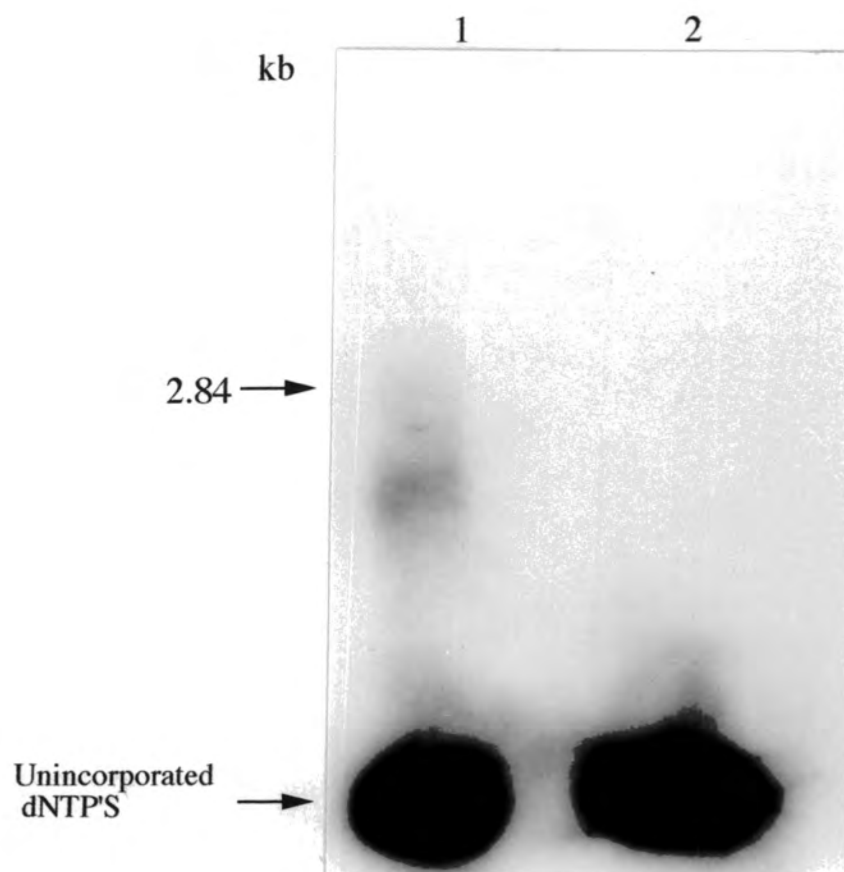

1
2
3
kDa
106
80
49.5
32.5
27.5
18

Figure 3.3 Analysis of 1st strand cDNA synthesis.

Using polyA+ RNA from *Coix* seeds, 1st strand cDNA was synthesised as described in section 2.19.1. An aliquot of this was electrophoresised on a 0.8% DNA agarose gel, see section 2.10.10 and exposed to X-ray film overnight (section 2.11).

Lane 1	3µl of first strand cDNA synthesis reaction.
Lane 2	Unincorpoated dNTP's



1
2
kb
2.84
Unincorporated
dNTP'S

Double stranded cDNA was synthesised from *Coix* seed polyA$^+$ RNA using a Pharmacia cDNA synthesis kit. 3μl was removed after 1st strand cDNA synthesis and electrophoresed in a 0.8% agarose/TAE gel to check the yield. Following the addition of EcoR I/Not I adaptors to the cDNA ends, the molecules were inserted into Eco RI-cut λgt11 and the religated vector packaged into phage particles using a Promega packaging mix. The packaged phage library was stored with a drop of chloroform at 4^0C and the titre of the phage library determined immediately.

3.5 Determination of the titre of the λgt11 cDNA expression library.

Determination of the number of recombinant and non-recombinant plaques present in the cDNA library is essential prior to amplification and screening of the library. λgt11 has no system that allows selection against parental bacteriophage, however, religation of parental phage is minimised by dephosphorylation of the λ arms. In this system non-recombinant phage are detected by their formation of blue plaques on strains of *E.coli* Y1090 and Y1088, in the presence of the inducer IPTG and the chromogenic substrate X-Gal, recombinant plaques are colourless.

In brief, 10μl from 1/10, 1/100 and 1/1000 dilutions from each of the three libraries were used to infect 50μl of Y1088 plating cells (section 2.19.3), mixed with 3ml of 0.8% top agar and plated out onto LB plates. Duplicate dilutions were plated onto LB plates containing X-Gal/IPTG. Following incubation at 42^0C overnight the number of plaque forming units (pfu) were counted for each dilution. The number of pfu's present on the 1/10 plates were to high to be counted accurately and it was decided that the 1/100 plates would give the most accurate pfu count. The numbers counted are illustrated below;

	-XGal/IPTG	+XGal/IPTG		No. of recombinant
		Blue	White	pfu/ml.
Ligation 1	346	7	304	3.04×10^6
Ligation 2	150	2	106	1.06×10^6
Ligation 3	289	40	241	2.41×10^6

Packaging of ligation 1 had been the most efficient and so it was decided to use this library for amplification and susequent screening using the antibody probe.

3.6 Amplification of the λgt11 cDNA expression library.

To amplify the library approximately 1.5 x 10^5 pfu were plated out on LB agar plates using Y1088 plating cells (section 2.19.4). The plates were incubated at 42^0C and plate stocks prepared. The titre of the plate stock was determined as 4.9 x 10^9 pfu/ml.

3.7 Immunoscreening of the *Coix* λgt11 cDNA library with α-amylase inhibitor antiserum.

3.7.1 Adsorption of antibodies against *E.coli* Y1090 antigens from the α-amylase-inhibitor antiserum.

It is possible that the antisera raised against the α-amylase inhibitor protein may have crossreacted with bacterial proteins, resulting in false positives and increased background when the library was screened. There are two main reasons for this; firstly non specific cross reaction between the antibodies and the *E.coli* proteins. Secondly, antibodies against bacterial proteins may be present in the preimmune serum due to previous bacterial infection of the host animal. To avoid this, cross reactive antibodies were adsorbed with *E.coli* Y1090 acetone powders prior to screening of the library to remove any anti-prokaryotic antibodies (section 2.15.4). To ascertain whether this had been succesful, a dot blot of *E.coli* Y1090 phage lysate (5mg/ml) was prepared, and probed with the 1/500 and 1/1000 dilutions of purified antisera. 1/10, 1/100, 1/1000 and 1/10,000 dilutions were probed with the α-amylase inhibitor antisera and the antigen-antibody complex detected with anti-rabbit IgG alkaline phosphatase (Figure 3.4A). The 1/10, 1/100 and 1/1000 dilution of the phage lysate gave a very weak positive reaction. The conclusion was that the antisera at both 1/500 and 1/1000 dilutions would not crossreact with *E.coli* antigens during the screening of the library.

3.7.2 Determination of antibody titre

There were several aims to these experiments;

1. To confirm the primary antibody's ability to detect at least nanogram quantities of antigen in an area the size of a small plaque.

2. To determine the appropriate concentration of the primary antibody, that is the greatest dilution that results in a strong positive reaction, so that possible background is minimilised.

3. To determine whether the primary antibody will react with the antigen after the transfer to nitrocellulose filters.

Figure 3.4 Dot blots with α-amylase inhibitor antibodies prior to immunoscreening.

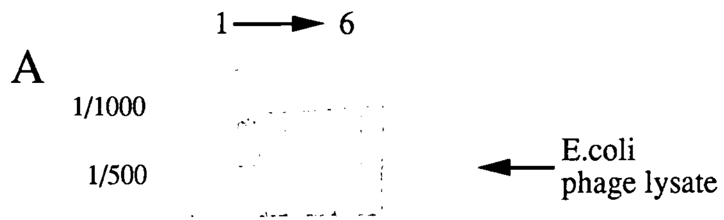
A. The α-amylase inhibitor antibodies were adsorbed with *E.coli* acetone powders prior to screening (2.15.4) and then used to probe dilutions of phage lysate (5mg/ml). The antigen -antibody complexes were detected with anti-rabbit IgG alkaline phosphatase.

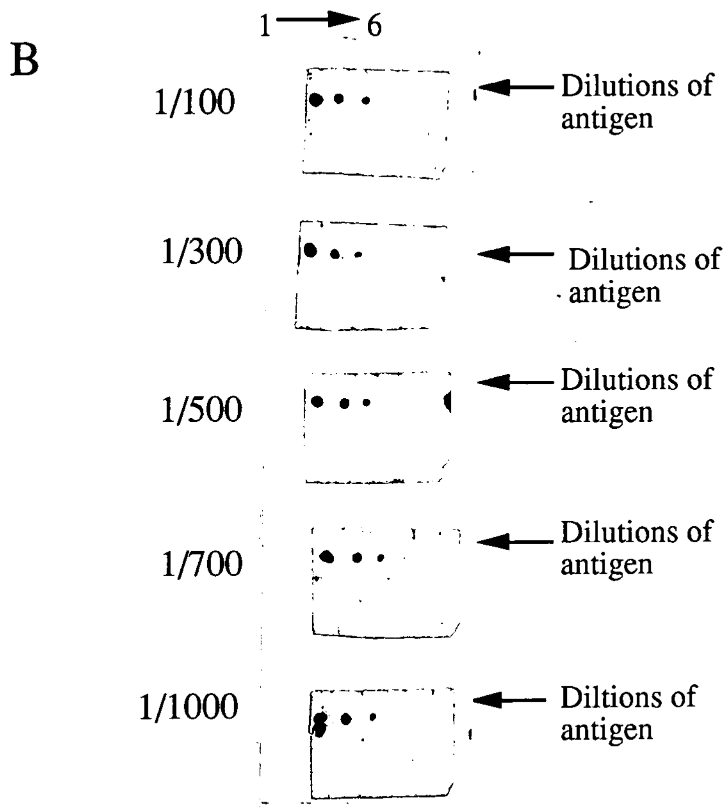
1. 1/10
2. 1/100
3. 1/1000
4. 1/10000 dilutions of *E.coli* phage lysate.

B. Dilutions of α-amylase inhibitor protein were spotted onto the nitrocellulose and probed with several dilutions of α-amylase inhibitor antibodies. The antigen-antibody complexes were detected with anti-rabbit IgG alkaline phosphatase.

1. 12ng of α-amylase inhibitor protein.
2. 4ng of α-amylase inhibitor protein.
3. 2ng of α-amylase inhibitor protein.
4. 0.1ng of α-amylase inhibitor protein.
5. 0.01ng of α-amylase inhibitor protein.
6. 0.001ng of α-amylase inhibitor protein.

The antibody titrations used were 1/100, 1/300, 1/500, 1/700 and 1/1000 as illustrated on the figures.


1 → 6
A
1/1000
1/500
E.coli
phage lysate


1 → 6
B
1/100
Dilutions of
antigen
1/300
Dilutions of
antigen
1/500
Dilutions of
antigen
1/700
Dilutions of
antigen
1/1000
Diltions of
antigen

12, 4, 2, 0.1, 0.01, 0.001ng quantities of the α-amylase inhibitor protein were spotted onto nitrocellulose strips. Using alkaline phosphatase-conjugated secondary antibody, antigen-primary antibody complexes were visualised as described in section 2.15.2. The result are shown in Figure 3.4B. There was a subtle colour contrast between the reactions obtained with different dilutions of primary antibody. Strong positive reactions were obtained for the 1/1000 dilution of antibody and so the library was screened using this dilution. The minimum amount of antigen detected was 2ng, which is sensitive enough to screen an expression library.

The amount of antigen that is deposited during a plaque lift incubation varies with each fusion protein based on its expression and stability in the host call. Succesful screening have been carried out with positives containing 30-60pg of immunoreactive material per plaque. Whereas some plaques contain as much as 200-800pg of fusion protein (Sambrook *et al.*, 1989).

3.7.3 Library screening.

A total of 600,000 pfu of the λgt11 expression library were plated onto a lawn of *E.coli* Y1090 plating cells and screened with a 1/1000 dilution of α-amylase inhibitor antisera as described in section 2.20. 3 positives were obtained. Agar plugs, containing the putative positive plaques, were isolated and secondary screened as described in 2.20.3 None of the three primary screen plaques were true positives.

3.7.4 Characterization of the α-amylase inhibitor antisera

M.Ary had previously characterised the α-amylase inhibitor antiserum as crossreacting with the 26,400Da α-amylase inhibitor protein under denaturing conditions as described in section 3.2.1. This antiserum had failed to crossreact with any proteins expressed in the *Coix* λgt11 cDNA library. Therfore, the antiserum was characterised again by probing crude total protein extracts from *Coix* seeds with the antiserum.

Total protein crude extract was prepared from *Coix* seeds (mid-late stages of maturation) (section 2.16) and separated by SDS-PAGE (12.5% running gel, 5% stacking gel) under denaturing conditions (2.15.1). Following transfer to nitrocellulose, the proteins were probed with a 1/500 dilution of the antiserum using alkaline phosphatase-conjugated anti-rabbit IgG as the secondary antibody (2.15.2). This Western blot is illustrated in Figure 3.5. Two major bands were visualised following the colour reaction with molecular weights of 68,000 and 64,000Da, this result was found to be reliable following several repeats of this experiment.

This contradicted the findings of M. Ary who had reported 2 immunopositive proteins both of approximately Mr 26,400Da.

3.8 Immunoscreening the λgt11 cDNA expression library with wheat germ endochitinase antiserum.

Prior to screening the *Coix* λgt11 cDNA library, the endochitinase antiserum was characterised by probing total protein crude extracts of leaf and seeds of *Coix* (section 2.15.1 and 2.15.2). Proteins which cross reacted with the antibody were found in both leaf and seed extracts (Figure 3.6). Two proteins were identified from seed extracts which had molecular weights of 29 and 35Da. 4 leaf proteins corresponding to molecular weights of 42, 40, 35 and 28Da cross reacted with the antibody.

400,000 pfu from the λgt11 expression library were plated onto *E.coli* Y1090 cells and screened for positive cDNA clones with a 1/500 dilution of the endochitinase antibody (section 2.20). Antibodies against *E.coli* antigens were removed from the endochitinase antibody prior to screening (section 2.15.4). 5 positives were obtained following the primary screen. By comparison of the filters with the master plate, 5 plugs were isolated containing the positive plaques which contained approximately 10 plaques per plug, as individual plaques could not be isolated. Following secondary screening, 3 of the original 5 positive cDNA clones remained positive. These were purified to homogeneity by tertiary screening (Figure 3.7) and the 3 cDNA inserts characterised .

3.9 PCR analysis of 3 immunopositive λgt11 clones

Using PCR, amplification of the immunopositive clones was carried out to asses the insert sizes. PCR was carried out on intact phage particles using primers to the λgt11 arms (section 5.12.1), and the products analysed on a 1% agarose gel (Figure 3.8). 820, 450 and 200bp PCR products were obtained from amplification of the cDNA inserts from λgt11 clones 2, 3, and 5 respectively.

Amplification of cDNA insert from clone pAT_2 was a positive control. This was succesfully amplified to generate a PCR product of approximately 450bp, which was as expected (personal communication Dr. A. Tommie).

3.10 Subcloning cDNA inserts

The cDNA inserts were excised from the λgt11 vector and subcloned into pUC 19 so that the inserts could be sequenced and characterised.

Figure 3.5 Western blot of *Coix* seed protein extracts probed with α-amylase inhibitor immunoserum

Crude protein extracts from seeds of *Coix* were seperated by SDS-PAGE and transferred to a nitrocellulose support (sections 2.15.1) *Coix* seed proteins were probed with antiserum raised against the *Coix* α-amylase inhibitor (gift of M. Ary, Rothemstead Experimental Station) as described in section 2.15.2.

Visualisation of proteins in lanes 1-3, following transfer to nitrocellulose is by staining with Ponceau S. Alkaline phosphatase-conjugated anti-rabbit IgG was used as a secondary anyibody.

Lane 1	High molecular weight markers
Lane 2	Low molecular weight markers
Lane 3	Crude total protein extracts from *Coix* seeds
Lane 4	Western blot of crude total protein extracts from *Coix* seeds Two immunopositive proteins were obtained with Mr of 68,000 and 64,000Da

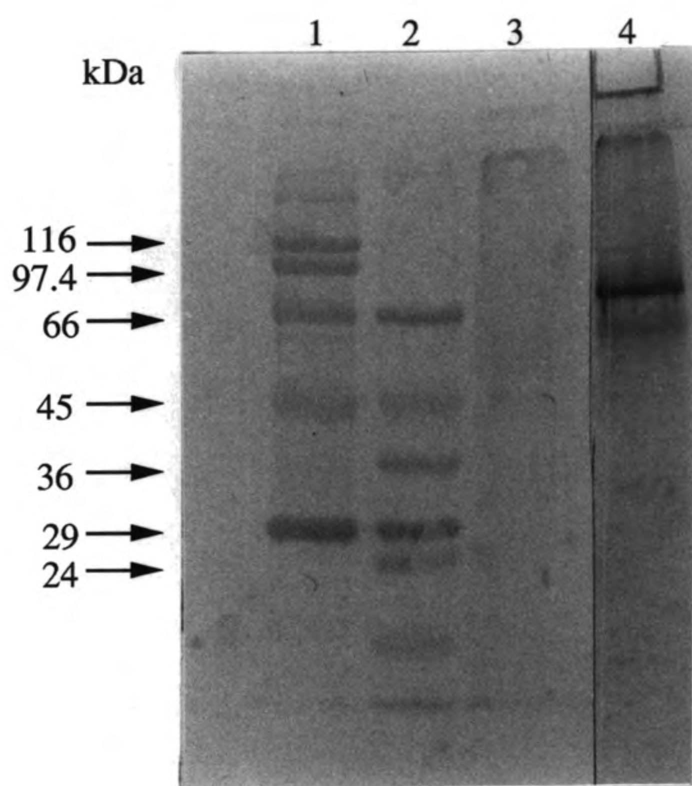

1
2
3
4
kDa
116
97.4
66
45
36
29
24

Figure 3.6 Western blot of *Coix* seed and leaf protein extracts probed with endochitinase immunoserum.

Crude total protein extracts from *Coix* seed and leaf tissue were seperated by SDS-PAGE and transferred to nitrocellulose (section 2.15.1 and 2.15.2). The proteins were probed with a 1/500 dilution of wheat germ endochitinase immunoserum and immunopositive proteins visualised using alkaline phosphatase-conjugated anti-rabbit IgG as a secondary antibody.

Lane 1	High molecular weight markers
Lane 2	Low molecular weight markers
Lane 3	Crude protein extracts from *Coix* seed 2 immunopositive proteins, Mr as estimated by SDS-PAGE to be 35,000 and 29,000Da.
Lane 4	Crude protein extracts from *Coix* leaf 4 immunopositive proteins, Mr as estimated by SDS-PAGE to be 42,000, 40,000, 35,000 and 29,000Da.

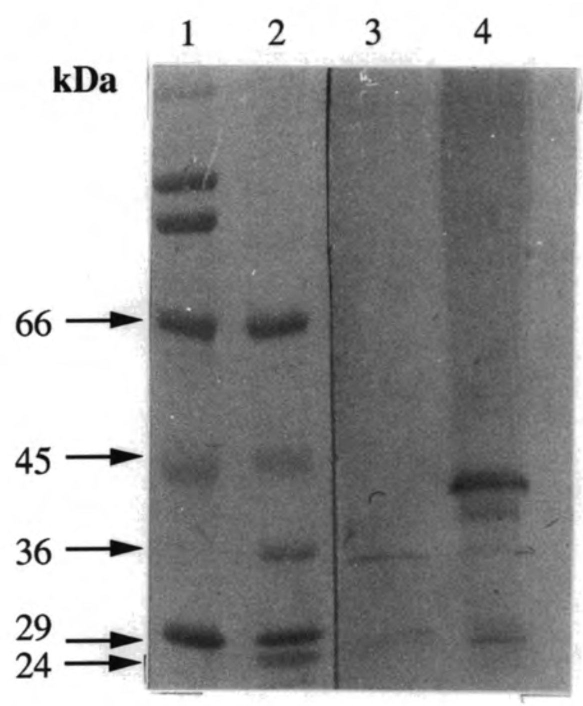

1
2
3
4
kDa
66
45
36
29
24

Figure 3.7 Tertiary screen of 3 immunopositive λgt11 clones

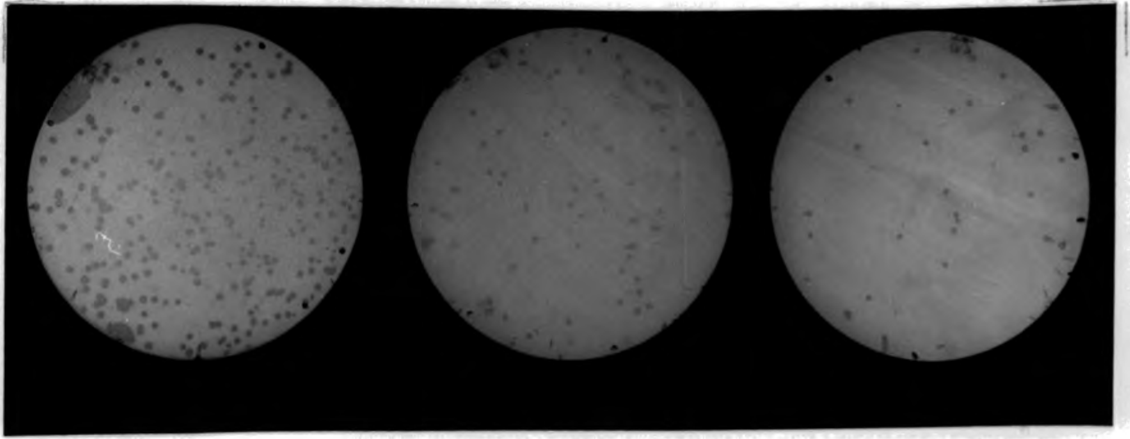
3 λgt11 clones were immunopositive following secondary screening of a *Coix* seed λgt11 cDNA library with wheat germ endochitinase immunoserum. To purify these clones to homogeneity, tertiary screens were carried out on the secondary plugs (section 2.20.3). The results of the tertiary screen are illustrated here.

A	λgt11 clone 2
1)	10^{-2} dilution of positive plaque
2)	10^{-3} dilution of positive plaque
3)	10^{-4} dilution of positive plaque

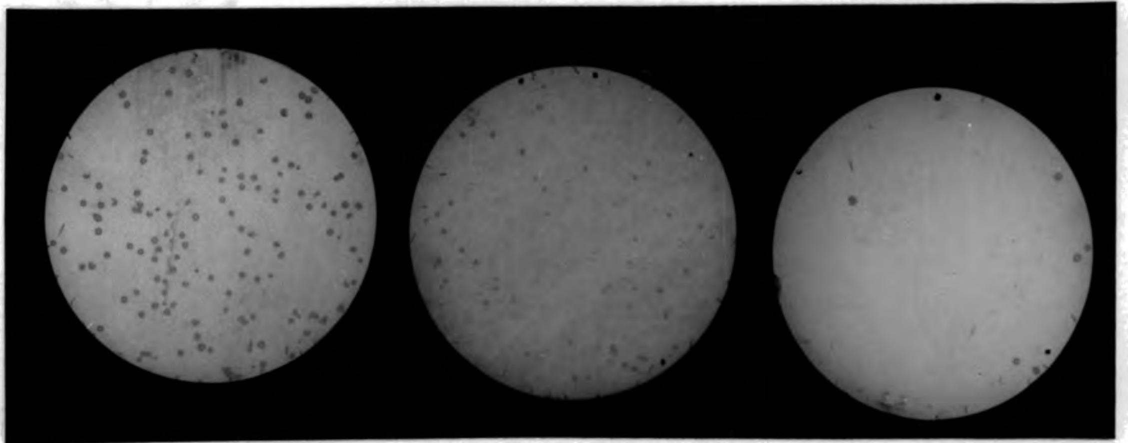
B	λgt11 clone 3
1)	10^{-2} dilution of positive plaque
2)	10^{-3} dilution of positive plaque
3)	10^{-4} dilution of positive plaque

C	λgt11 clone 5
1)	10^{-2} dilution of positive plaque
2)	10^{-3} dilution of positive plaque

A 1 2 3



B 1 2 3



C 1 2

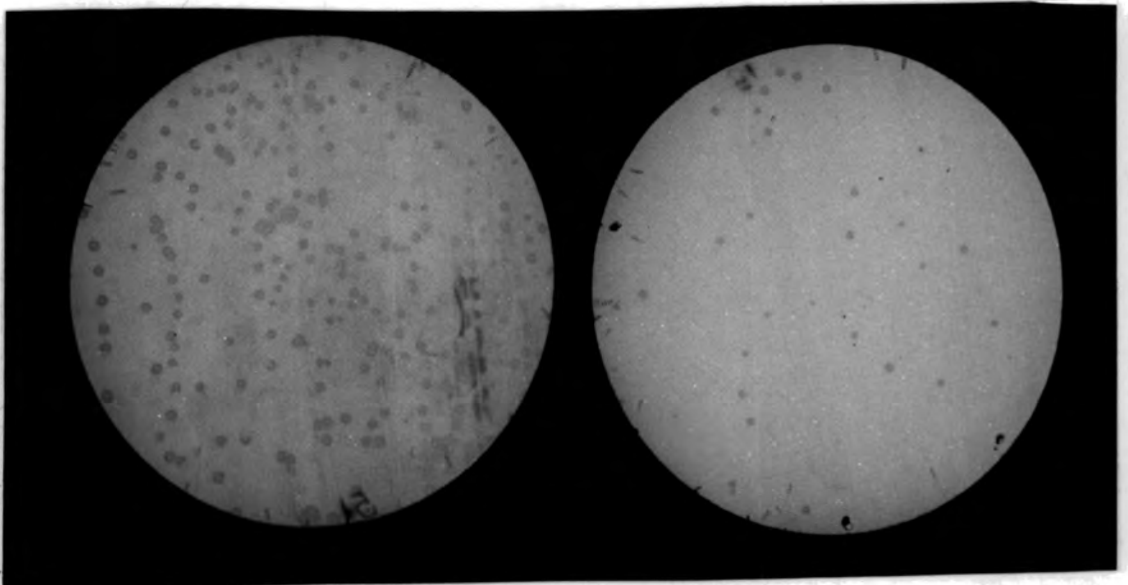
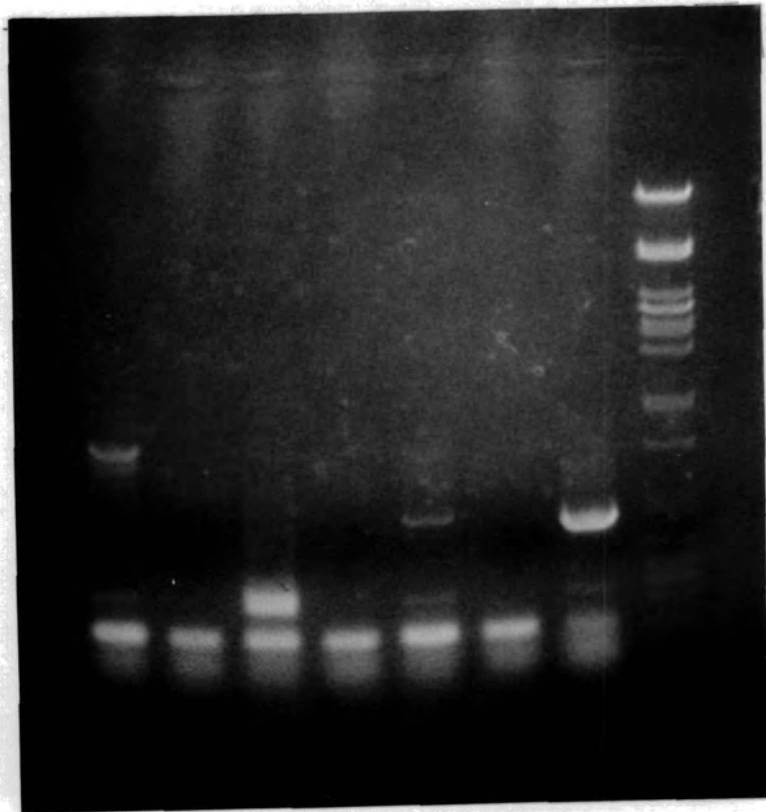


Figure 3.8 DNA electrophoresis of PCR products following amplification of 3 immunopositive λgt11 clones.

3 immunopositive clones were isolated following the immunoscreening of a *Coix* seed λgt11 library with wheat endochitinase immunoserum (section 3.8). PCR was carried out on the phage particles using primers to the λgt11 arms, to assess the insert size of these 3 clones (section 5.12.11).

Lane 1	PCR products following amplification from λgt11 clone 2	820bp
Lane 3	PCR products following amplication from λgt11 clone 5	200bp
Lane 5	PCR products following amplification from λgt11 clone 3	410bp
Lane 7	PCR products following amplification from λgt11 clone pAT_2	450bp
Lane 8	λPst I DNA markers	

Lane 2, 4 and 6 Blank


1
2
3
4
5
6
7
8
bp
820
450
410
200

2ug of phage DNA (section 2.22) from each of the 3 positive clones was restiction digested with EcoR1 (section 2.10.3) and the digest products ligated to EcoR1 restricted pUC19 (2.10.7). *E.coli* XL1-Blue was transformed with the ligation mix, and positive transformants selected on tetracycline/ampicillin LB plates, with XGal and IPTG so that recombinants could be selected (section 2.9).

Recombinant DNA was prepared from 10 transformants (section 2.8.1) and restriction analysed with Eco R1 to check for cloned inserts in the pUC19 vector. All 3 cDNA inserts from the λgt11 subclones were succesfully subcloned into pUC19. Subclones of the cDNA inserts from the 3 λgt11 immunopositive clones 2, 3 and 5 were called pDF2.2, pDF3.17 and pDF5.1 respectively.

3.11 Sequence analysis of 3 immunopositive cDNAs

Sequencing of the 3 insert DNA's was carried out using an Applied Biosystems automatic sequencer as described in (section 2.27.2). Clones pDF2.2, pDF5.1 and pDF3.17 were sequenced in both directions using the forward and reverse sequencing primers (-21m13 and M13RP1).

The EcoRI/Not 1 adaptors defining the ends of the cDNA were excluded from any further sequence analysis of the 3 inserts. 6 phase translations were carried out on the sequence data using a DNA analysis computer programme called DNA Strider, to identify any open reading frames. Nucleotide and deduced amino acid sequnces were analysed for similiarity with characterised sequences using the EMBL database at the Daresbury facility (section 2.28).

3.11.1 Sequence analysis of pDF5.1

The entire cDNA was sequenced, which corresponded to 155bp, and a 36 amino acid open reading frame identified, see Figure 3.9 The nucleotide sequence and the deduced amino acid sequences were analysed by comparison with EMBL data bases (section 2.28), no significant sequences similiarities were found in either case.

3.11.2 Sequence analysis of pDF3.17

The entire cDNA was sequenced which corresponded to 198 bp and contained an open reading frame which was translated into 41 amino as illustrated in Figure 3.10. To identify the clone pDF3.17, the deduced amino acid sequence was analysed by comparison with proteins in the EMBL data base (section 2.28). The deduced amino acid sequence was 91.667% similiar and 86.111% identical to a 22,000Da α-zein precursor protein (Geraghty *et*

Figure 3.9 Nucleotide sequence and deduced amino acid sequence of cDNA clone pDF5.1

pDF5.1 was isolated following immunoscreening with endochitinase antiserum, of a *Coix* seed λgt11 cDNA library (section 3.8).

The cDNA insert was sequenced in both directions and the coding region translated into amino acid sequence by DNA Strider (section 2.28).

```
  1 ATT TTC CTT TTC AAT TCA ATT GTT CAT  27
  1 Ile Phe Leu Phe Ile Ser Ile Val His   9

 28 CTT CAG GTG ATT TGT TCG CAT ATA TTG  54
 10 Leu Gln Val Ile Cys Ser His Ile Leu  19

 55 TCA CCC ATG GTG ATG GAT GAC ACT TTT  81
 20 Ser Pro Met Val Met Asp Asp Thr Phe  29

 82 ATT ACC CAT CGC CGC GCT AAA TGA cta 108
 30 Ile Thr His Arg Arg Ala Lys *

109 aac tag ctt agc tcg cca gcg gct ata 135


136 tta cat cat tat tat tac ag
```

al., 1981), and 73.684% similiar and 63.158% identical to a 19,000Da α-zein precursor protein, both from maize. Additionally, the protein is 86.111% similiar and 77.778 % identical to a prolamin seed storage protein from Sorghum, called Kafirin *psk*8.

2 putative polyadenylation signals, AATAAT, GATAAA are present in the 3' untranslated region of the pDF3.17 sequence. This cDNA insert appears to be the 3' end of the full length cDNA encoding for a seed storage protein.

Coix seed and leaf RNA was probed with the pDF3.17 cDNA insert to confirm that it was expressed in seed tissue and not in leaf, as expected for seed storage proteins (Figure 3.11).

Alignments of the pDF3.17 amino acid sequence with the maize and sorghum proteins were carried out using the GAP protein alignment package as described in materials and methods. The alignments are illustrated in Figure 3.12.

3.11.3 Sequence analysis of pDF2.2

The size of the cDNA insert had previously been identified by PCR as 820bp, 329bp were sequenced using the M13 forward primer and 327bp using the M13 reverse primer. These sequnces did not overlap, and so an estimated 164bp remained unsequenced. An initial observation was that the cDNA cloning adaptors were not complete and that although the EcoR1 sites were present, the Not 1 sites were not. It is relatively common for the adaptor sequences to be incomplete following subcloning and characterisation of the subclone proceeded (personal communication L. Edwards and J. Davies, University of Durham).

Open reading frames were identified for both sequences, 109 amino acids for the forward sequence and 112 amino acids for the reverse sequence (section 2.28), Figures 3.13 A and B respectively. Both open reading frames were in the same frame, which implied that the cDNA insert contained one complete open reading frame. Sequencing of the insert was not completed as it was obvious that the cDNA did not encode for the α-amylase inhibitor.

To identify pDF2.2 the deduced amino acid and nucleotide sequences were analysed by comparison with sequences in the EMBL databases as described in materials and methods 2.28. At the protein level, both amino acid sequences are virtually 100% identical to a stretch in a 699 amino acid open reading frame in the *E.coli* transposable element Tn1000 (EMBL ascesion number S21899, personal communication Broom *et al.,* 1992). At the nucleotide level, the forward sequence is 99.392% similiar and 99.392% identical and the reverse sequence is 98.516% similiar and 98.516% identical to a stretch of sequence in the 2097bp open reading frame of the *E.coli* transposable element Tn1000.

Figure 3.10 Nucleotide sequence and deduced amino acid sequence of cDNA clone pDF3.17

pDF3.17 was isolated following immunoscreening with endochitinase antiserum, of a *Coix* seed λgt11 cDNA library (section 3.8).

The cDNA insert was sequenced in both directions and the coding region translated into amino acid sequence by DNA Strider (section 2.28).

Putative polyadenylation signals are underlined.

```
  1 TTG GTT GCC ACC TTC CTA CAG CAG CAG   24
  1 Leu Val Ala Thr Phe Leu Gln Gln Gln    9

 25 CAA CGG CAA CTA CTG CCA TTC AAC CAA   51
 10 Gln Arg Gln Leu Leu Pro Phe Asn Gln   18

 52 ATG TCT TTG ATG AAC CCT GCC TTG TCG   78
 19 Met Ser Leu Met Asn Pro Ala Leu Ser   29

 79 TGG CAG CAA CCC ATC GTT GGA GGT GTC  105
 30 Trp Gln Gln Pro Ile Val Gly Gly Val   38

106 GGC TTC TAG att aaa tat gag ttg tac  135
 39 Gly Phe  *

136 ttg ata aaa aag ctc tca tgc cgg ctt  164

165 gtg caa ctt cct aga aat aat caa tat  193

194 att g
```

Figure 3.11 Northern blot of *Coix* seed and leaf RNA probed with cDNA pDF3.17.

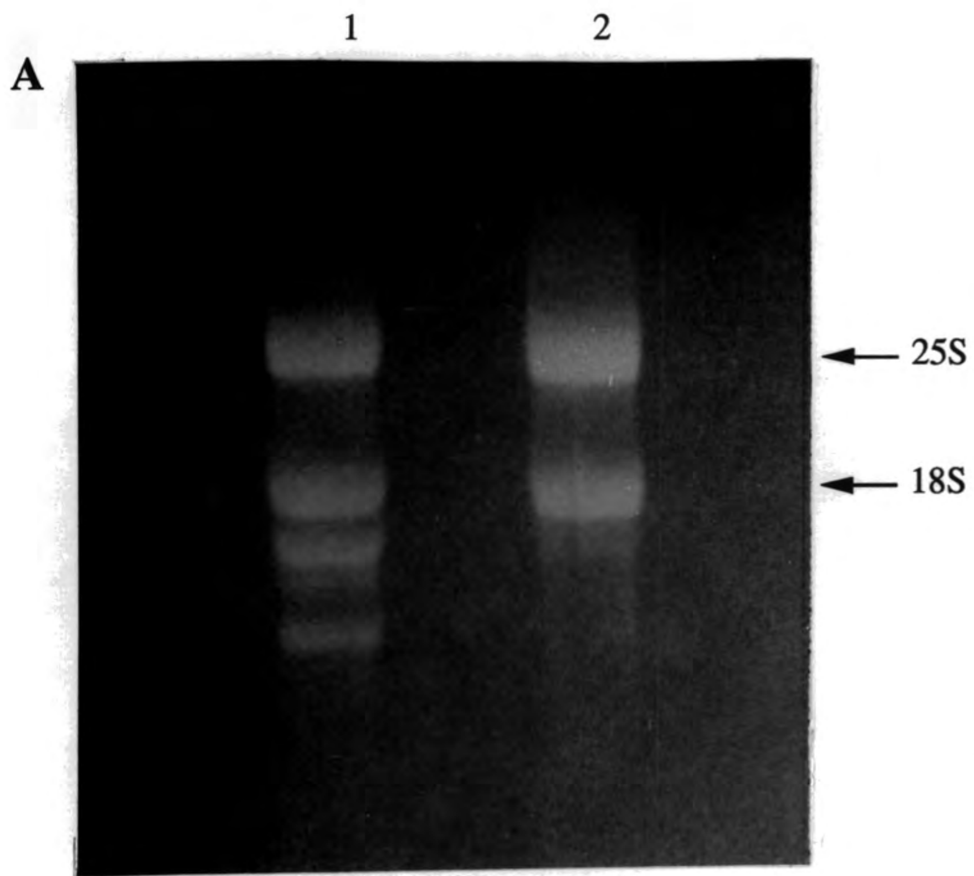
RNA from *Coix* seed and leaf tissue was seperated by formaldehyde agarose gel electrophoresis and transferred to a nylon support (section 2.13.1 and 2.13.2). The RNA was probed with the endlabelled cDNA insert from pDF3.17 (section 3.11.2).

A. Agarose gel electrophoresis of RNA.

Lane 1	Leaf RNA
Lane 2	Seed RNA

B. Northern blot.

Lane 1	Leaf RNA
Lane 2	Seed RNA


1
2
A
25S
18S

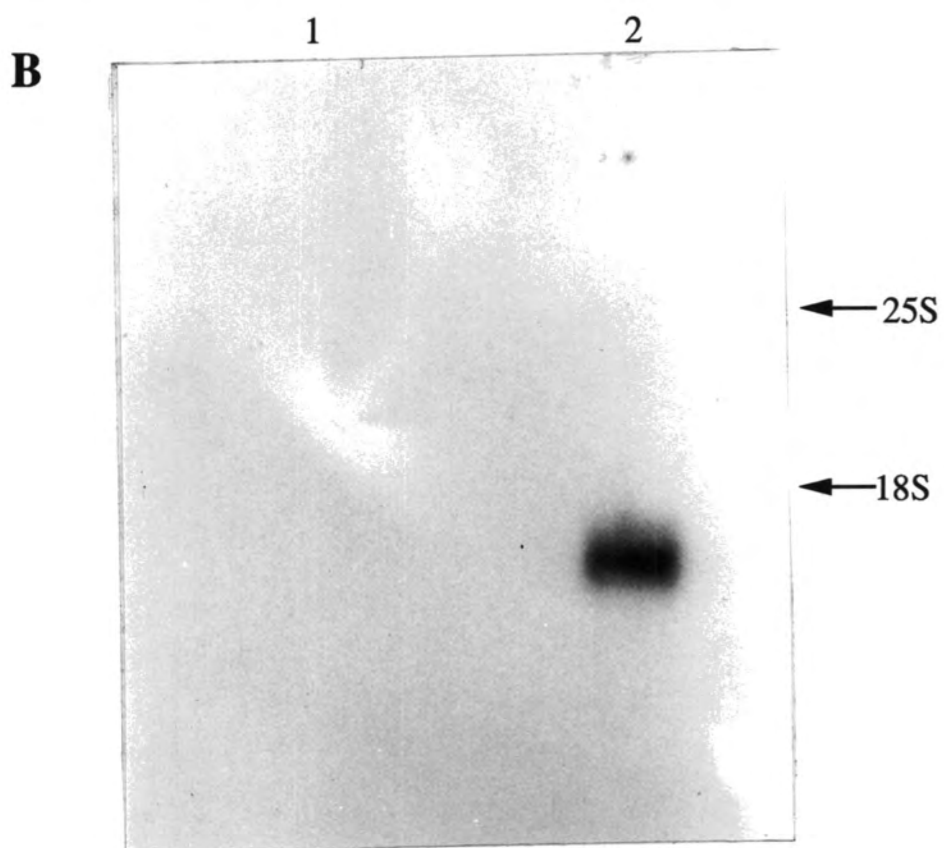

1
2
B
25S
18S

Figure 3.12 Alignment of the deduced amino acid sequence of cDNA clone pDF3.17 with maize and sorghum seed storage proteins

Alignments of these amino acid sequences were carried out using the GAP programme (section 2.28).

A. GAP alignment of cDNA clone pDF3.17 with the maize 22kD α-zein.

B. GAP alignment of cDNA clone pDF3.17 with the maize 19kD α-zein.

C. GAP alignment of cDNA clone pDF3.17 with the sorghum kafirin protein

A

```
GAP of: zein.3  check: 7489  from: 1  to: 38

zein.2

to: zea5_maize.swissprot  check: 3229  from: 1  to: 234

ID   ZEA5_MAIZE     STANDARD;      PRT;   234 AA.
AC   P08416;
DT   01-AUG-1988 (REL. 08, CREATED)
DT   01-AUG-1988 (REL. 08, LAST SEQUENCE UPDATE)
DT   01-AUG-1988 (REL. 08, LAST ANNOTATION UPDATE)
DE   ZEIN-ALPHA PRECURSOR (19 KD) (CLONE GZ19AB11). . . .

Symbol comparison table: /exel/gcg/gcgcore/data/rundata/nwsgappep.cmp
CompCheck: 1254

         Gap Weight:  3.000      Average Match:  0.540
      Length Weight:  0.100   Average Mismatch: -0.396

            Quality:   35.6             Length:    234
              Ratio:  0.937               Gaps:      1
 Percent Similarity: 73.684   Percent Identity: 63.158

zein.3 x zea5_maize.swissprot December 3, 1992  15:39  ..

                                         .
                                         .
                                         .
                     .         .         .         .         .
     1 ............................LVATFLQQQQ............ 10
                                   .|||| |.|
   151 PFNQLAALNSPAYLQQQQLLPFSQLAGVSPATFLTQPQLLPFYQHVAPNA 200
                     .         .         .
    11 ......RQLLPFNQMSLMNPALSWQQPIVGGVGF 38
             .||||||||:.| |||: :||||:||. |
   201 GTLLQLQQLLPFNQLALTNPAVFYQQPIIGGALF 234
```

B

```
GAP of: zein.3  check: 7489  from: 1  to: 38

zein.2

to: zeav_maize.swissprot  check: 9641  from: 1  to: 122

ID   ZEAV_MAIZE     STANDARD;      PRT;   122 AA.
AC   P05815;
DT   01-NOV-1988 (REL. 09, CREATED)
DT   01-NOV-1988 (REL. 09, LAST SEQUENCE UPDATE)
DT   01-NOV-1988 (REL. 09, LAST ANNOTATION UPDATE)
DE   ZEIN-ALPHA (22 KD) (CLONE B49) (FRAGMENT). . . .

Symbol comparison table: /exel/gcg/gcgcore/data/rundata/nwsgappep.cmp
CompCheck: 1254

         Gap Weight:  3.000      Average Match:  0.540
      Length Weight:  0.100   Average Mismatch: -0.396

            Quality:   45.5             Length:    124
              Ratio:  1.197               Gaps:      1
Percent Similarity: 91.667    Percent Identity: 86.111

zein.3 x zeav_maize.swissprot December 3, 1992  15:41  ..

                                   .
                                   .
                                   .
                .         .         .         .         .
     1 ....................................LVATFLQQQQRQLL 14
                                           |||.|||||  |||
    51 AYLQQLLPFNQLTVSNSAAYLQQRQQLLNPLVVANPLVAAFLQQQ..QLL 98

                .         .
    15 PFNQMSLMNPALSWQQPIVGGVGF 38
       |:||:|||||||||||||||. |
    99 PYNQFSLMNPALSWQQPIVGGAIF 122
```

C

```
 GAP of: zein.3  check: 7489  from: 1  to: 38

zein.2

 to: kaf1_sorbi.swissprot  check: 4229  from: 1  to: 269

ID   KAF1_SORBI     STANDARD;      PRT;   269 AA.
AC   P14690;
DT   01-APR-1990 (REL. 14, CREATED)
DT   01-APR-1990 (REL. 14, LAST SEQUENCE UPDATE)
DT   01-APR-1990 (REL. 14, LAST ANNOTATION UPDATE)
DE   KAFIRIN PGK1 PRECURSOR. . . .

 Symbol comparison table: /exel/gcg/gcgcore/data/rundata/nwsgappep.cmp
 CompCheck: 1254

          Gap Weight:  3.000      Average Match:  0.540
       Length Weight:  0.100   Average Mismatch: -0.396

             Quality:   41.4            Length:    271
               Ratio:  1.089              Gaps:      1
 Percent Similarity: 86.111   Percent Identity: 77.778

 zein.3 x kaf1_sorbi.swissprot December 3, 1992  15:42  ..

                                         .
                                         .
                                         .
                        .         .         .         .         .
       1 .................................LVATFLQQQRQLLPFN 17
                                          |||.|||||  || .||
     201 QLLPINQLALANTDAYLQQQLLPVNPLVVANPLVAAFLQQQ..QLSSFN 248

                        .         .
      18 QMSLMNPALSWQQPIVGGVGF 38
         |:||:||||||||||:||. |
     249 QISLVNPALSWQQPIIGGAIF 269
```

Figure 3.13 Nucleotide sequence and deduced amino acid sequence of cDNA clone pDF2.2

pDF2.2 was isolated following immunoscreening with endochitinase antiserum, of a *Coix* seed λgt11 cDNA library (section 3.8).
The cDNA insert was partially sequenced in both directions using M13 forward and reverse primers and the coding regions from each translated into amino acid sequence by DNA Strider (section2.28).

A. pDF2.2 sequence information with M13 forward primer

B. pDF2.2 sequence information with M13 reverse primer

A

```
  1 GCA CAA GGT GAC ATT TTA AGA AAG TGT AGG   30
  1 Ala Gln Gly Asp Ile Leu Arg Lys Cys Arg   10

 31 CTT GTC GCA AAG GAG TAT TTA GAT GAA AAT   60
 11 Leu Val Ala Lys Glu Tyr Leu Asp Glu Asn   20

 61 AAC CCA GAA GAA TCA ATT GGT GAT CTT CAA   90
 21 Asn Pro Glu Glu Ser Ile Gly Asp Leu Gln   30

 91 TTC AAT TTG AAT ATC TCA GAA ATA GAA AAT 120
 31 Phe Asn Leu Asn Ile Ser Glu Ile Glu Asn   40

121 AAT ATA GTA TCA CTT CTT GAA CGC TCA GAC 150
 41 Asn Ile Val Ser Leu Leu Glu Arg Ser Asp   50

161 AGG AAA GTT GTC ATA TTA ATG GAT AAG CTA 180
 51 Arg Lys Val Val Ile Leu Met Asp Lys Leu   60

181 GAT GAG GCG TAT GAC CCG GAT AAT ATA GGA 210
 61 Asp Glu Ala Tyr Asp Pro Asp Asn Ile Gly   70

211 ATT GGA ATC ATT GCA GGT CTA GCA TAT GCA 240
 71 Ile Gly Ile Ile Ala Gly Leu Ala Tyr Ala   80

241 TCT ATT GAA TTA AAT CAA AAA GCA AAA TGC 270
 81 Ser Ile Glu Leu Asn Gln Lys Ala Lys Cys   90

271 ATT CGT CCA ATA ATT TTT TTA AGG GAT AAT 300
 91 Ile Arg Pro Ile Ile Phe Leu Arg Asp Asn 100

301 ATA TTT GGG TCG CTA TCA AAG GAA GAT CC
101 Ile Phe Gly Ser Leu Ser Lys Glu Asp
```

B

```
  1 AAG CCG ATG TTG CAA TTT ACC CTT TAC CGG. 30
  1         Met Leu Gln Phe Thr Leu Tyr Arg   8

 31 CCC AGG GAT TTA CTA TCA TTG TTG AAT GAA  60
  9 Pro Arg Asp Leu Leu Ser Leu Leu Asn Glu  20

 61 GCT TTT TTT TCC GCA TTC AGA GAG AAT AGA  90
 21 Ala Phe Phe Ser Ala Phe Arg Glu Asn Arg  30

 91 GAA ACT ATC ATA AAC ACT GAC CTA GAA TAT 110
 31 Glu Thr Ile Ile Asn Thr Asp Leu Glu Tyr  40

111 GCA GCC AAG TCA ATT TCC ATG GCC AGA CTT 140
 41 Ala Ala Lys Ser Ile Ser Met Ala Arg Leu  50

141 GAA GAT CTC TGG AAA GAG TAT CAG AAG ATC 170
 51 Glu Asp Leu Trp Lys Glu Tyr Gln Lys Ile  60

171 TTT CCT TCA ATA CAG GTT ATA ACT AGT GCA 200
 61 Phe Pro Ser Ile Gln Val Ile Thr Ser Ala  70

201 TTT CGT AGC ATT GAA CCT GAA TTA ACA GTT 230
 71 Phe Arg Ser Ile Glu Pro Glu Leu Thr Val  80

231 TAT ACG TGC TTA AAA AAA ATA GAA GCA TCT 260
 81 Tyr Thr Cys Leu Lys Lys Ile Glu Ala Ser  90

261 TTC GAA TTA ATC GAA GAA AAT GGA GAT CCT 290
 91 Phe Glu Leu Ile Glu Glu Asn Gly Asp Pro 100

291 AAA ATA ACG TCT GAA ATA CAG TTG TTA AAG 320
101 Lys Ile Thr Ser Glu Ile Gln Leu Leu Lys 110

321 GCA AGT G
111 Ala Ser
```

GAP alignments were carried out on both nucleotide and protein sequences with the Tn1000 open reading frame (section 2.28), Figures 3.14 and 3.15 respectively.

Figure 3.14 Alignment of the nucleotide sequence of pDF2.2 with an open reading frame in *E.coli* Tn 1000

Alignments of both forward and reverse nucleotide sequences were carried out using the GAP programme (section 2.28). Sequences were aligned with a 2097 bp reading frame from the *E.coli* transposable element Tn1000 (EMBL ascession number S21899).

A. Alignment of 327bp nucleotide sequence obtained from forward primer DNA sequencing.

B. Alignment of 327bp nucleotide sequence obtained from reverse primer DNA sequencing.

a

```
GAP of reverse of: forwarf  check: 7178  from: 1  to: 329

forwarddd

 to: x60200.em_ba  check: 9466  from: 1  to: 5982

ID   ECTN1000   standard; DNA; PRO; 5982 BP.
XX
AC   X60200;
XX
DT   15-AUG-1991 (Rel. 29, Created)
DT   15-AUG-1991 (Rel. 29, Last updated, Version 4) . . .

 Symbol comparison table: /exel/gcg/gcgcore/data/rundata/nwsgapdna.cmp
 CompCheck: 6876

         Gap Weight:  5.000      Average Match:  1.000
      Length Weight:  0.300   Average Mismatch:  0.000

            Quality:  327.0             Length:   5982
              Ratio:  0.994               Gaps:      0
 Percent Similarity: 99.392   Percent Identity: 99.392

 forwarf x x60200.em_ba    November 20, 1992  12:23  ..

                                .
                                .
                                .
                   .         .         .         .         .
  329 ..............................................GGAT 326
                                                    ||||
 1401 CCAATGCAACCTTATGACTTGACCCTCTATATTTCTCGAGTAATCAGGAT 1450
                   .         .         .         .         .
  325 CTTCCTTTGATAGCGACCCAAATATATTATCCCTTAAAAAAATTATTGGA 276
      |||||||||||||||||| |||||||||||||||||||||||||||||||
 1451 CTTCCTTTGATAGCGACCTAAATATATTATCCCTTAAAAAAATTATTGGA 1500
                   .         .         .         .         .
  275 CGAATGCATTTTGCTTTTTGATTTAATTCAATAGATGCATATGCTAGACC 226
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 1501 CGAATGCATTTTGCTTTTTGATTTAATTCAATAGATGCATATGCTAGACC 1550
                   .         .         .         .         .
  225 TGCAATGATTCCAATTCCTATATTATCCGGGTCATACGCCTCATCTAGCT 176
      |||||||||||||||||||||||||||||| |||||||||||||||||||
 1551 TGCAATGATTCCAATTCCTATATTATCCGGTTCATACGCCTCATCTAGCT 1600
                   .         .         .         .         .
  175 TATCCATTAATATGACAACTTTCCTGTCTGAGCGTTCAAGAAGTGATACT 126
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 1601 TATCCATTAATATGACAACTTTCCTGTCTGAGCGTTCAAGAAGTGATACT 1650
                   .         .         .         .         .
  125 ATATTATTTTCTATTTCTGAGATATTCAAATTGAATTGAAGATCACCAAT 76
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 1651 ATATTATTTTCTATTTCTGAGATATTCAAATTGAATTGAAGATCACCAAT 1700
                   .         .         .         .         .
   75 TGATTCTTCTGGGTTATTTTCATCTAAATACTCCTTTGCGACAAGCCTAC 26
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 1701 TGATTCTTCTGGGTTATTTTCATCTAAATACTCCTTTGCGACAAGCCTAC 1750
                   .         .         .         .         .
   25 ACTTTCTTAAAATGTCACCTTGTGC......................... 1
      |||||||||||||||||||||||||
 1751 ACTTTCTTAAAATGTCACCTTGTGCAGAATTCCATTTTTCAAATGTTCA 1800
                                .
                                .
                                .
```

b) GAP of reverse of: reversec check: 4260 from: 1 to: 337

reverseb

to: x60200.em_ba check: 9466 from: 1 to: 5982

```
ID   ECTN1000   standard; DNA; PRO; 5982 BP.
XX
AC   X60200;
XX
DT   15-AUG-1991 (Rel. 29, Created)
DT   15-AUG-1991 (Rel. 29, Last updated, Version 4) . . .
```

Symbol comparison table: /exel/gcg/gcgcore/data/rundata/nwsgapdna.cmp
CompCheck: 6876

```
         Gap Weight:  5.000      Average Match:  1.000
      Length Weight:  0.300   Average Mismatch:  0.000

            Quality:  332.0             Length:   5982
              Ratio:  0.985               Gaps:      0
 Percent Similarity: 98.516   Percent Identity: 98.516
```

reversec x x60200.em_ba   November 20, 1992 13:53 ..

```
                                 .
                                 .
                                 .
                   .         .         .         .         .
 337 .....................................CACTTGCCTTTAA 325
                                          |||||||||||||
 901 CCAACAAATCCTACACTATACAAACTTTGGAGAATTCCACTTGCCTTTAA 950
                   .         .         .         .         .
 324 CAACTGTATTTCAGACGTTATTTAGGATCTCCATTTTCTTCGATTAATT 275
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 951 CAACTGTATTTCAGACGTTATTTAGGATCTCCATTTTCTTCGATTAATT 1000
                   .         .         .         .         .
 274 CGAAAGATGCTTCTATTTTTTTAAGCACGTATAAACTGTTAATTCAGGT 225
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1001 CGAAAGATGCTTCTATTTTTTTAAGCACGTATAAACTGTTAATTCAGGT 1050
                   .         .         .         .         .
 224 TCAATGCTACGAAATGCACTAGTTATAACCTGTATTGAAGGAAAGATCTT 175
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1051 TCAATGCTACGAAATGCACTAGTTATAACCTGTATTGAAGGAAAGATCTT 1100
                   .         .         .         .         .
 174 CTGATACTCTTTCCAGAGATCTTCAAGTCTGGCCATGGAAATTGACTTGG 125
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1101 CTGATACTCTTTCCAGAGATCTTCAAGTCTGGCCATGGAAATTGACTTGG 1150
                   .         .         .         .         .
 124 CTGCATATTCTAGGTCAGTGTTTATGATAGTTTCTCTATTCTCTCTGAAT 75
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1151 CTGCATATTCTAGGTCAGTGTTTATGATAGTTTCTCTATTCTCTCTGAAT 1200
                   .         .         .         .         .
  74 GCGGAAAAAAAAGCTTCATTCAACAATGATAGTAAATCCCTGGGCCGGTA 25
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1201 GCGGAAAAAAAAGCTTCATTCAACAATGATAGTAAATCCCTGGGCCGGTA 1250
                   .         .         .         .         .
  24 AAGGGTAAATTGCAACATCGGCTT.......................... 1
     |||||||||||||||     ||||
1251 AAGGGTAAATTGCAAACATCGCTTAAAACCATTCCTCCCTTTAAGATCAT 1300
                                 .
                                 .
                                 .
```

Figure 3.15 Alignment of deduced amino acid sequence of pDF2.2 with an open reading frame in *E.coli* Tn 1000

Alignments of both forward and reverse amino acid sequences from pDF2.2 were carried out using the GAP programme (section 2.28). Sequences were aligned with a 699 amino acid reading frame from the *E.coli* transposable element Tn1000.

A. Alignment of 109 amino acid sequence deduced from M13 forward primer DNA sequencing.

B. Alignment of 112 amino acid sequence deduced from M13 reverse primer DNA sequencing.

```
      1 MELNITSKSNPFGDTTAENDKKMLNAFIETADFRTLIETDDR  42

     43 TIVVGRRGTGKSALFIQLNEHWKKDKKILILSFSPDDSQIIG  84

     85 FRSMLKPFTGSFNLARAATRLLWRYAMLMEIASYISSHYKLS 126

a                                 AQGDILRKCRLVAKEYLDENNPEE
    127 SQISSETLLNEHLKKWNSAQGDILRKCRLVAKEYLDENNPEE 168

        SIGDLQFNLNISEIENNIVSLLERSDRKVVILMDKLDEAYDP
    169 SIGDLQFNLNISEIENNIVSLLERSDRKVVILMDKLDEAYEP 210

        DNIGIGIIAGLAYASIELNQKAKCIRPIIFLRDNIFRSLSKE
    211 DNIGIGIIAGLAYASIELNQKAKCIRPIIFLRDNIFGSLSKE 252

        D
    253 DPDYSRNIEGQVIRLHWDWAQLLMLSAKRMKVAFKLDIEKDQ 296

                                    LQFTLYRPRDLLSLLNEAFFSA
    297 RVWDRCTADDLKGRNGFKRCLQFTLYRPRDLLSLLNEAFFSA 338

        FRENRETIINTDLEYAAKSISMARLEDLWKEYQKIFPSIQVI
    339 FRENRETIINTDLEYAAKSISMARLEDLW-EYQKIFPSIQVI 380

        TSAFRSIEPELTVYTCLKKIEASFELIEENGDPKITS
    381 TSAFRSIEPELTVYTCLKKIEASFELIEENGDPKITSEIQLL 422

    423 KASGILQSLYSVGFVGIRDKNTSSYSFCHDGRTPDKKGFESN 464

    465 EKLLIHPCYWLGLNLNRNALAPEEAEEINDEYDINIISDNSA 506

    507 IRNKTIGQITTHLDQIPIGNEGATEFEQWCLDALRIVFASHL 548

    549 TDIKSHPNGNAVQRRDIIGTNGGKSDFWKRVLEDYKTRQVVF 590

    591 DAKNFEELGPSEYRQLQSYLTGPYGKLGFIINRDESEVLKSG 632

    634 KDLDWTKEMYQSHNSLIIKLPAKYISKLLQKLRNPEKHDAID 674

    675 RQMGKLLTLYETSYMAIKSTQKKRRK
```

3.12 DISCUSSION.

The aim of this research was to isolate the cDNA clone encoding for an α-amylase inhibitor from the seeds of *Coix*. The strategy was to immunoscreen a λgt11 cDNA library, with two antibodies raised against the *Coix* α-amylase inhibitor and a wheat germ endochitinase.

3.12.1 Immunoprecipitation of *Coix* seed translation products with α-amylase inhibitor immune serum.

Ary *et al.* (1989) determined the molecular weight of the native *Coix* α-amylase inhibitor to be 52,600Da comprising of two near identical subunits of 26,400Da. Reticulocyte lysates do not carry out secondary modifications such as glycosylation or cleavage of signal sequences. Lack of these modifications affects the electrophoretic mobility of the polypeptides and can make identification of specific immunoprecipitation products difficult (Berger and Kimmel, 1987). As no information was available regarding the extent of these properties in the α-amylase inhibitor, it was futile to estimate the effect of the lack of these modifications on the electophoretic mobility of the primary translation product. One can assume that the primary translation product is larger than the mature protein due to the presence of the signal peptide sequence. The signal peptide sequence on average is approximately 20 amino acids in length. Initially, the immunprecipitation product of 27,000Da was thought to be the most likely candidate for the α-amylase inhibitor primary translation product. The smaller 17,000Da and 18,000Da products could be degradation products or incomplete translation products due to premature termination.

However the 27,000Da protein was only immunoprecipitated with 1/5 and 1/50, and not with a 1/500 dilutions of α-amylase inhibitor immunoserum. This suggested that these results may be artifactual.

Subsequently, two immunopositive proteins with Mr 68,000 and 64,000Da were detected following probing of *Coix* seed total protein extracts with the α-amylase inhibitor immunoserum. Ary *et al.* (1989) estimated the M*r* of the α-amylase inhibitor to be 26,400Da under similiar denaturing conditions. This anomaly suggests that the immunoserum does not cross react with the α-amylase inhibitor protein, and so brings into question the validity of the immunoprecipitation experiments.

3.12.2 Preparation of the *Coix* λgt11 library

The fact that the α-amylase inhibitor cDNA was not isolated form the λgt11 library may be a reflection of the library quality. The λgt11 library was oligo d(T) primed, this often results in

a bias of 3' end cDNAs being represented in the library. These clones will predominately express C-terminal epitopes. If the antibodies predominately recognise the N terminal epitopes not present in the library, the cDNA will not be detected. λZapII libraries (Stratagene) are now the preferred choice when constructing cDNA libraries, they are random and oligo d(T) primed, providing an improved representation of mRNA species.

3.12.3 The abundance of the α-amylase inhibitor mRNAs in the total transcript population.

The frequency at which a cDNA clone of a particular mRNA appear in the cDNA library is generally proportional to the abundance of that species in the mRNA population. A typical eukaryotic cell contains 10^6 mRNA molecules, transcribed from about 15,000 different genes (Berger and Kimmel, 1987).

Most genes are members of a low abundance class, and are represented by about 20 mRNA copies per cell. A smaller number are expressed at higher levels (medium abundance) and a few mRNA's accumulate to a few percent of cellular mRNA or even more in some cases.

Thus the recombinant DNA expression library must contain sufficient numbers of individual recombinants to ensure that the DNA sequence of interest appears in the library. When screening libraries with antibody probes the presence of multiple copies of DNA is important. This is because antigen coding sequences are generally only expressed in appropriate translation and orientation frame. DNA sequences of interest can usually be detected 5 to 10 fold more frequently with nucleic acid probes than with antibody probes.

Unfortunately, the exact abundance of the *Coix* α-amylase inhibitor mRNA in the seed is not known, and so the number of clones which should ideally be screened could not be predicted. The α-amylase inhibitor mRNA's are certainly not rare transcripts and are thought to comprise up to 1% of cellular RNA's although this is variable between species and varieties (personal communication Dr. Hilder and Dr. Gatehouse, University of Durham).

600,000pfu of amplified and 400,000pfu of non amplified library were screened with the α-amylase inhibitor and the endochitinase immunoserum respectively. The number of recombinants screened should be sufficient to isolate the α-amylase inhibitor, given that the mRNA is thought to be of medium abundance.

It is feasible to construct a cDNA library containing 10^5 to10^7 recombinant in a λ vector starting with a few mg of polyA$^+$ RNA. The *Coix* λgt11 cDNA library contained approximately 10^6 recombinant clones which should be large enough to contain sequences

representing even the rarest mRNA's. For example, a recombinant library of >5000 different cDNAs is required to have a >99% probability of finding a specific sequence that represents a 0.1% of the mRNA population. For most expression libraries constructed in λgt11, the equivalent is at least 6 fold greater, since only one of every six clones is a template for the appropriate peptide. One may need to screen > 10^6 clones to identify a rare mRNA using an antibody (Berger and Kimmel, 1987).

It is possible to estimate the number of clones that must be screened in order to be arbitrarily certain of encountering at least one copy of the desired clone using the following formulae (Sambrook *et al.,* 1989);

$$N=\frac{\ln(1-P)}{\ln(1-n)}$$

N=The number of clones required for screening.

n=The fractional proportion of the total mRNA poulation represented by a single mRNA species.

p=probability which is usually 99%.

3.12.4 The α-amylase inhibitor antigen

The characteristics of the antigen under study can have a major effect on the probability of a clone being detected. Some eukaryotic proteins fused to *lacZ* and expressed in *E.coli,* are unable to adopt their correct conformation and may not bind to the antibodies (Harlow and Lane, 1988). However, although some epitopes are complicated topological structures formed by the folding of noncontigous regions of one or more polypeptide chains, others consist of tracts of adjacent amino acids from a single polypeptide chain. Such localised epitopes can be formed in foreign proteins expressed in bacteria even though the protein as a whole may be malfolded and non functional. In addition fragments of proteins can form simple epitopes and will retain at least part of their immunological reactivity. Polyclonal antisera normally react with many different epitopes thus maximising the chances of positive interaction between the antigen expressed in *E.coli* and the antibodies. It is conceivable that the α-amylase inhibitor could not fold adequately to form appropriate epitopes and so could not be detected by the antibodies.

Additionally, the major antigenic determinants for a number of proteins lies within attached carbohydrate moiety. Glycosylation does not occur in *E.coli* , and so glycosylation of an

expressed fusion peptide will fail to occur in the same manner as in plant cells. This may result in a loss of antigenicity of the protein.

It is conceivable that the α-amylase inhibitor antigen is toxic to the *E.coli* host. This may prevent sufficient deposition of the fusion peptide for detection by the assay system.

3.12.5 The α-amylase inhibitor immunoserum

M.Ary had originally characterised the α-amylase inhibitor immunoserum as cross reacting with a *Coix* seed α-amylase inhibitor protein, M*r* approximately 26,400Da as estimated by SDS-PAGE under denaturing conditions (personnel communication). Attempts to characterise the immunoserum again have repeatedly shown that the immunoserum crossreacted strongly with 2 *Coix* seed proteins, Mr 68,000 and 64,000Da as estimated by SDS-PAGE under denaturing conditions. There is a significant size discrepancy between these two results. It is very unlikely that the α-amylase inhibitor was not denatured prior to SDS-PAGE as all protein samples were boiled for 2 minutes in sample buffer containing β-mercaptoethanol, which is standard procedure for protein denaturation. In general, differences in size estimation by SDS-PAGE should only vary by +/- 1% (personal communication Dr. K. Elborough). It is unlikely that the 68,000 and 64,000Da proteins are the *Coix* α -amylase inhibitor. This brings into question the actual identity of the immunoserum which was recieved from M.Ary.

Several attempts at screening the *Coix* seed λgt11 library with this α-amylase inhibitor immunoserum had been unsuccesful in obtaining immunopositive clones. Considering the strong imunopositive reaction obtained with the 68,000 and 64,000Da proteins in the Western blot of *Coix* seed proteins described above, it is surprising that cDNAs encoding for these two proteins were not immunopositive.

3.12.6 The wheat germ endochitinase immunoserum

4 immunopositive proteins were identified in leaf tissue, and 2 in seed tissue following probing of total protein extracts with endochitinase immunoserum. A protein of M*r* 29,000 was identified in seed but not in leaf. This was of similiar size to the seed specific α-amylase inhibitor protein, which has Mr 26,400Da under similiar SDS-PAGE conditions. Another protein which was immunopositive in seed and leaf tissue, has Mr 35,000Da as estimated by SDS-PAGE. This protein along with the 42,000, 40,000 and 28,000Da proteins in leaf tissue were initially believed to be endochitinases.

Following characterisation of the 3 immunopositive cDNA inserts isolated by screening of the λgt11 library with endochitinase immunoserum, it seems likely that one of the immunopositive proteins in the seed extracts is a seed storage protein, named α–coixin (section 3.10). As α-acoixins are seed specific, it is unlikely to be the 35,000Da protein as it is present in leaf tissue. That is assuming that both 35,000Da immunopositive proteins are the same protein. Therefore it is possible that the 29,000Da protein is the α-coixin. The deduced amino acid sequence of α-coixin is similiar to 2 α-zein proteins with *Mr* 22,000Da and 19,000Da (Geraghty *et al.,* 1981).

3.12.7 Identification of three immunopositive λgt11 cDNA clones

Three immunopsitive clones were isolated following immunoscreening of a *Coix* seed λgt11 library with wheat germ endochitinase immunoserum. Sequence information for all 3 cDNAs will be discussed.

3.12.7.1 pDF5.1

pDF5.1 is a 155bp insert with a deduced 38 amino acid open reading frame. No significant homology was found at either the protein or nucleotide level following extensive searches within the EMBL data bases. The AT ratio for the cDNA insert is 62% overall and 60% for the coding region which is not unusual for a plant gene. Additionally, there are no polyadenylation sites present in the 3' untranslated region. Due to time constraints and the fact that this was not the α-amylase inhibitor cDNA, attempts to isolate the full length cDNA clone were not made. This may have yielded more sequence information and may have enabled identification of this cDNA. A Western blot of *Coix* seed protein probed with the wheat germ endochitinase anti serum, had identified two immunopositive proteins, Mr 35,000 and 29,000Da. It is likely that this cDNA encodes for one of these proteins.

3.12.7.2 pDF3.17

pDF3.17 is a 198bp cDNA insert which contains a 41 amino acid open reading frame which has been identified as being 91.667% similiar and 86.111% identical to a 22kD α-zein precursor protein. In *Coix* these zein like proteins have been termed coixins (Targon, 1992). The nucleotide sequence of a cDNA clone encoding γ-coixin from *Coix lachryma jobi* has been isolated by Leite *et al.* 1991. Due to time constraints and the fact that this was not the *Coix* α-amylase inhibitor, attempts to isolate the full length cDNA were not pursued.

3.12.7.3 pDF2.2

Although pDF2.2 was not sequenced in its entirety, sequence information was available for approximately 330bp from both ends. The nucleotide and deduced amino acid sequences for both sequences were analysed and found to be virtually 100% identical to an open reading frame in the *E.coli* transposable element Tn1000. This extent of similiarity, particularly at the nucleotide level, implies that the putative cDNA insert was infact a fragment of the *E.coli* transposable element. This was confirmed by the failure of the end labelled pDF2.2 E.coRI insert to hybridise to Northern blots of seed and leaf RNA(results not presented).

It is known that *E.coli* strain Y1090 (host for λgt11), carried Tn1000 in its F' episome. Following digestion of Tn1000 with EcoRI, a 800bp fragment is generated, this is approximately the same as the estimated cDNA insert size of λgt11 clone 2 (as estimated by PCR amplification of the cDNA insert). One explanation for the cloning anomaly of pDF2.2 is that when attempting to subclone the cDNA insert from λgt11 into pUC19, transformants will have contained a mixture 800bp inserts i.e. cDNA insert and Tn1000 EcoRI fragment. Unfortunately they were indistinguishable following restriction digest of subclones with EcoRI and the cloning artifact was sequenced and characterised instead of the cDNA insert from λgt11 clone 2.

3.12.8 Why did an endochitinase immunoserum cross react against these cDNA expression products.

The wheat germ endochitinase antibodies crossreacted with epitopes present in the expression products of pDF5.1 and pDF3.17. Neither of these expression products were endochitinases or α-amylase inhibitors. There are several reasons why the immunoserum crossreacted with these proteins.

The original endochitinase antigen may have been contaminated with several proteins, for example, with a wheat seed storage protein. Consequently, seed storage protein antibodies could have been present in the immunoserum sample. Additionally, if these contaminant proteins were immunodominant, they would constitute the majority of antibodies in the immunoserum (Harlow and Lane, 1988). When assayed, this would result in a stronger signal of cross reaction betwen these antibodies and contaminant proteins when compared to detection of endochitinases.

Unfortunately, protein sequence information for the wheat germ endochitinase (Molano *et al.*, 1979) is not available, and so motif comparisons of the endochitinase with the cDNA proteins could not be carried out. This would have determined whether stretches of amino acids in the cDNA expression products could form epitophes similiar to those in the wheat germ endochitinase protein.

3.12.9 Further experiments.

The inserts from pDF5.1 and pDF3.17 could have been used as probes to screen the *Coix* λgt11 cDNA library to isolate the full length cDNA clones. The additional sequence information may enable identification of clone pDF5.1.

Additionally, both cDNAs could be used to screen a genomic library (Frichhauf *et al.*, 1983). A *Coix* EMBL 3 genomic library had been constructed during this work with the aim of isolating the α-amylase inhibitor genomic sequence, after isolation of the cDNA clone. The genomic clones coresponding to pDF5.1 and pDF3.17 would enable identification of gene regulatory sequences, promoter analysis and possibly information regarding the position of introns and flanking sequences. In particular, sequence comparisons between pDF3.17 full length cDNA and genomic clones with other known seed storage protein genes, such as the α-zeins would be interesting.

Southern blot information, following the probing of *Coix* genomic DNA with pDF5.1 and pDF3.17 would reveal whether the genes are single or multi copy in the *Coix* genome. It is extremely likely that pDF3.17, which has been characterised as an α-coixin is a multi copy gene.

An alternative approach to isolating the α-amyalse inhibitor gene is by heterologous probing of the cDNA library. The cDNAs encoding α-amylase inhibitors from bean (Altabella and Chrispeels, 1990), wheat (Garcia-Moroto, 1990) and barley (Leah and Mundy, 1989; Rasmussen and Johansson, 1992) have been isolated. Unfortunately, these clones are not commonly available due to their wide commercial applications. Oligonucleotides could be designed from highly conserved regions of these α–amylase inhibitors nucleotide sequences. Thus, oligonucleotide screening of a *Coix* cDNA library may be a viable approach for isolation of the α-amylase inhibitor cDNA and genomic clones.

Oligonucleotide screening of the cDNA library was previously attempted using a 109 base oligonucleotide (462) encoding a region of the *Coix* α -amylase inhibitor. This oligonucleotide had originally been designed for expression in pGEX3X to produce an α-amylase inhibitor peptide for antibody production. Unfortunately, screening did not result in the isolation of the appropriate clone (results not presented). This was possibly due to the fact that when designing the oligonucleotide *E.coli* codon usage was used in base positions of redundancy. The reason being that the oligonucleotide was to be expressed in *E.coli* XL1-Blue.

Additionally, pDF2.2 is at present being subcloned, with the aim of sequencing and identifying the cDNA insert.

CHAPTER 4

PRODUCTION OF POLYCLONAL ANTIBODIES AGAINST AN α-AMYLASE INHIBITOR FUSION PROTEIN.

4.1 Introduction

The experiments in this section describe the raising of polyclonal antisera which could be used to immunoscreen a *Coix* seed λgt11 expression library, with the aim of isolating the cDNA(s) encoding for the *Coix* α-amylase inhibitor protein. Immunoscreening of a *Coix* seed expression library had been attempted previously, as described in Chapter 3, with polyclonal antiserum (M.Ary, Rothemstead Experimental Station) raised against the isolated α-amylase inhibitor protein (Ary *et al.*, 1989). These antibodies had failed to isolate positive cDNAs encoding for the α-amylase inhibitor gene and so a new approach to developing α-amylase inhibitor antibodies was undertaken. As pure α-amylase inhibitor protein was not available, an α-amylase inhibitor synthetic peptide fusion protein, produced by a bacterial expression system, was used as the antigen.

Several strategies were available for obtaining an antigen. For example, protein purification of the α-amylase inhibitor from seed material was an option. This was decided against as expertise was available in molecular techniques rather than protein purification.

The use of synthetic peptides as immunogens is an important technique for the preparation of antibodies specific for previously uncharacterised proteins (Sutcliffe *et al.*, 1980; Lerner *et al.*, 1981, 1982, 1984; Walter *et al.*, 1980; Walter, 1986; Doolittle, 1976). The synthesised peptides are purified and coupled to carrier proteins, and these conjugates used to immunize animals.

Alternatively, antibodies can be raised against antigen produced by overexpression of a cloned DNA in bacteria (reviewed in Carroll and Laughton, 1987; Marston, 1987). A wide variety of coding regions can be expressed in bacteria either on their own or as fusion proteins. There are a number of vectors available for the production of fusion proteins. Vectors producing β-galactosidase, anthranilate synthetase (trpE) and glutathione-S-transferase are the most common, but others are available. Some have inducible expression whereas others are fully constitutive.

The bacterial expression vector pGEX3X was chosen to synthesise a glutathione-S-transferase-α-amylase inhibitor bacterial fusion protein, to be used as an immunogen to raise polyclonal antiserum.

4.1.1 The pGEX vectors

The pGEX vectors (Smith and Johnson, 1988; Johnson *et al.*, 1989a) were designed so that foreign polypeptides could be expressed in *E. coli* in a form that allows them to be purified rapidly under non-denaturing conditions. Foreign polypeptides are expressed as fusions to the C-terminus of glutathione-S-transferase, a common 26,000Da cytoplasmic protein of eukaryotes. The GST gene used to generate the pGEX vectors was originally cloned from the parasitic helminth, *Schistosoma japonicum* (Smith and Johnson, 1988).

The GST "carrier" protein is vital for the production of good antibodies when the foreign protein is less than M*r* 3-5,000Da. The requirement for both an epitope and a class II-T cell receptor binding site imposes a minimal size limit on the immunogen molecule. Molecules smaller than this often bind to the surface antibodies on the B cell but may not have the suitable site for the simultaneous binding of a class II protein and T cell receptor. Physical coupling of small molecules to larger immunogenic molecules overcome this problem by providing the missing class II T cell receptor binding sites and allows induction of good antibodies (Harlow and Lane, 1988).

Following expression, the fusion proteins normally remain soluble within the bacteria and can be purified from lysed cells because of the affinity of the GST moiety for gluthathione, which is immobilised on agarose beads. Recovery of the fusion proteins is by elution at neutral pH with free reduced glutathione. Denaturing conditions are not required at any stage during purification, and so, foreign polypeptides may retain their functional activities and antigenicity. If necessary, the GST moiety can be removed from fusion proteins by cleavage with site specific proteases. In most cases the GST carrier does not compromise the antigenicity or functional activity of the foreign polypeptide.

4.1.2 Production of a GST-α-amylase inhibitor fusion peptide in bacteria

The three dimensional structure of the α-amylase inhibitor protein was unknown, and so prediction of the sequences which would give rise to epitopes was difficult. When choosing a peptide sequence for the production of antibodies several criteria can be considered (Harlow and Lane, 1988).

(i) If possible use more than one peptide, unfortunately this can make the cost of raising antibodies by this method unaffordable for many researchers.

(ii) Use the carboxy or amino-terminal ends of the protein sequence if it contains hydrophilic amino acids.

(iii) Proline residues are more likely to be exposed on the surface of the protein than other residues, and form epitopes for antibody recognition.

Even after satisfying these properties it is still not indicative of a good antigen because when the peptide is coupled to the carrier it has a different local environment than in the original protein.

A computer aided analytical method was used when designing the α-amylase inhibitor antigen. This allowed some insight into the 3-dimensional structures coded in the primary sequence These methods offer the advantage of quickly derived useful information about protein antigenic sites and other sites of interaction. Most of the methods available yield similiar information. Particularly useful are hydrophilicity/hydophobicity plotting methods which offers ways of determining protein surfaces, interaction sites and folding patterns.

Antigenic sites are simply the areas on the surface of a protein that can form good contacts with antibodies specific for that protein. It has been observed that many of these antigenic sites share certain characteristics in common. For example, one of the first features noticed of an antigenic site is that they usually contain a preponderance of charged and polar amino acids (Sela and Mozes, 1969). Most investigations suggest that predicted hydrophilic sites are likely regions of high antigenicity and may include secondary structures such as α-helices, β-strands and β-turns in their analysis.

The hydrophilicity methods often cited include Hopp and Woods (1981); Hopp (1986); Rose and Roy (1980); Kyte and Doolittle (1982). Whereas, secondary structures are mostly predicted by the methods of Chou and Fasman (1984) or Garnier *et al.* (1978).

Protein secondary structures (α-helices, β-strands and β-turns) all depend on the hydrophilic or hydrophobic nature of local protein chain segments, and are therefore related to the hydrophilicity methods. It can therefore be concluded that the secondary structure methods all incorporate a substantial influence of hydrophilicity and hydrophobicity on the resulting predictions.

The method used to determine the major antigenic sites of the *Coix* α-amylase inhibitor was one which combines a method like that of Chou-Fasman in which numerical values for amino acids are repetitively averaged over the length of the polypeptide chain, with a set of values expressing the relative hydrophilicity of each amino acid. These values are termed antigenic indexes. The most antigenic regions of the α-amylase inhibitor peptide sequence were predicted by constructing a Chou-Fasmann plot of the partial peptide sequence. This assigns an antigenic index to each of the amino acid residues in the partial peptide sequence.

A plot of all the peptide sequences together was constructed, although many of the sequences are non contiguous.

Two complementary oligonucleotide were designed which encoded for the α-amylase inhibitor peptide sequence. As the oligonucleotide sequence was to be expressed in *E.coli* XL-1Blue, the codon preference for expression in *E.coli* was taken into account when designing this oligonucleotide sequence.

The two complementary oligonucleotides were designed to form BamH1 and EcoR1 overhangs, to facilitate cloning into BamHI/ EcoRI digested pGEX3X. An extra two bases were added to each oligonucleotide sequence between the restriction site and the start of the peptide encoding region, to allow the correct reading frame for expression in the pGEX3X vector.

4.1.3 Production of GST-α-amylase inhibitor immunoserum

Using the GST-α-amylase inhibitor fusion protein as an antigen, the strategy was to raise polyclonal antibody in mice. Polyclonal antibodies are preferred for the initial screening of cDNA expression libraries, since potentially they contain antibodies which will recognise the maximum number of epitopes presented by the recombinant antigen. In contrast monoclonal antibodies recognise only one epitope which may not be expressed by the fusion protein. In essence polyclonal antisera increase the probability of obtaining a positive signal following screening.

The choice of using the GST-fusion protein as an antigen, rather than cleave the fusion and raise an antibody against the α−amylase inhibitor peptide alone was due to the small molecular size of the peptide. The α-amylase inhibitor peptide contained 33 amino acids which corresponds to a M*r* of approximately 3,630Da. By remaining linked to GST, the integrity of the peptide molecule once injected into the mice was stabilised.

The two main areas involved in producing a good immune response to an antigen, are choice of antigen and antigen injection conditions. The injection conditions involve choice of animal, the dose and form of antigen, the use of adjuvants, the route and number of injections, and the period left between each injection (Harlow and Lane, 1988).

The strategy was to immunize 6 three month old female mice (Balb/c) with an additional one mouse as a non immune control. Mice were used in preference to rabbits due to the relatively shorter immunization times required to raise antiserum of a suitable titre. Ideally, suitable antibodies can be raised in 6 weeks in mice.

After primary injection of antigen, antibodies are detected in serum from around 7 days after detection and persist at a low level for a few days, typically reaching a peak around day 10. Primary responses are normally very weak.

The response to the second injection is very different. The number of B cells bearing antigen-specific cell surface antibodies increases exponentially after the second injection, resulting in a peak between days three and four. Antibodies in the serum are also detectable at this time but peak levels are achieved around days 10-14. Typically high levels of antibody persist for about 2-4 weeks after the second injection. The response to the third and following injections broadly mimics that of the second injection with higher titres of antibody being reached (Harlow and Lane, 1988).

4.2 Design of 2 complementary oligonucleotides encoding an α-amylase inhibitor peptide.

The partial peptide sequence of the *Coix* α-amylase inhibitor (Figure 4.1) was analysed to construct a Chou-Fasmann plot which is illustrated in Figure 4.2. Additionally, antigenic indexes were calculated for individual amino acids which are presented in Figure 4.3. The peptide sequence chosen to design the oligonucleotides was from a contiguous sequence of 33 amino acids containing amino acid residues with the highest antigenic index. The sequence and postion of this peptide sequence in relation to the α-amylase inhibitor partial peptide sequence is illustrated in Figure 4.1. The sequences of the two complementary oligonucleotides (462 and 463) designed to encode for the α-amylase inhibitor peptide are shown in Figure 4.4.

Oligonucleotides 462 and 462 were synthesised (section 2.29), and the yield and integrity of the two 109bp oligonucleotides analysed by acrylamide gel electrophoresis. (1μg of each oligonucleotide was electrophoresed on a 12% acrylamide gel at 105V for 2 hours, using a BioRad mini protean vertical gel system. λPst 1 DNA markers were electrophoresed along side for size reference). The gel was visualised following staining with ethidium bromide and is shown in Figure 4.5. The 109 base oligonucleotide was the most abundant sequence for each of the synthesis reactions and so synthesis had been successful.

4.3.1 Subcloning 2 complementary oligonucleotides into pGEX3X.

The two complementary oligonucleotides were annealed following incubation at 95^{0}C for 3 minutes, and then ligated into BamH1/EcoR1 restricted vector (2.10.3) at molar ratios of 1:1, 1:3, 1:10 (vector:insert), overnight at 4^{0}C (section 2.10.7), and used to transform *E.coli* XL-

Figure 4.1 The partial peptide sequence of the *Coix* α-amylase inhibitor illustrating the position of the position of the peptide to be expressed in pGEX3X.

The peptide sequences marked in bold type contains the amino acids which have the highest antigenic index

Residue number

	1 ... 50
Bean	EQCGRQAGGALCPGGNCCSQFGWCGSTTDYC-PG-CQSQC--GGPSPAPT
Potato	QNCGSQGGGKACASGQCCSKFGWCGNTNDYCGSGNCQSQCPGGGPGPGPG
Tobacco	EQCGSQAGGARCASGLCCSKFGWCCNTNDYCGPGNCQSQCPGG-PTPPGG
Coix	CCSKFGYCGLTDAY

	51 ... 100
Barley	*SVSSIVSRAQFDRMLLHRNDGATQAKGFYTYDAFVAAAAAFPGFGRTGS
Bean	-DLSALISRSTFDQMLKHRNDGACPAKGFYTYDAFIAAAKAYPSFGNTGD
Potato	GDLGSAISNSMIDQMLKHRNLNSCQCKNFYSYNAFINAARSFPGFGTSGD
Tobacco	GDLGSIISSSMFDQMLKHRNDNACQGKGFYSYNAFINAARSFPGFGTSGD
Coix	NFYTGQLTS

	101 ... 150
Barley	ADARK****
Bean	TATRKREIAAFLGQTSHETTGGWATAPDGPYAWGYC-FYRER-NPSTYC-
Potato	INARKREIAAFFAGTGHLTTGGWASAPDGPYAWGYC-FLRERGNPGDYC-
Tobacco	TTARKREIAAFFAQTSHETTGGWATAPDGPYAWGYCWL-REQGSPGDYCT
Coix	FAHVTHETG **NNAYCD**

	151 ... 200
Bean	SATPQFPCAPGQQYYGPGPIQISWNYNYCQCGRAIGVDLLNKPDLVATDS
Potato	PPSSQWPCAPGRKYFGPGPIQISHNYNYGPCGRAIGVDLLNNPDLVATDP
Tobacco	PS-GQWPCAPGRKYFGRGPIQISHNYNYGPCGRAIGVDLLNNPDLVATDP
Tobacco	***VATDP
Coix	**PSKTQKPCAAGKKYYGRGPIQISXNYNY**GPCGRAIGMDGLGNPDRVAQDA

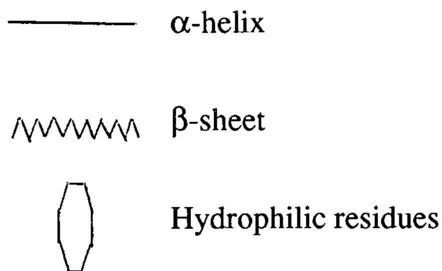
Barley	**MTAQPPKPSSHAVIAGQWSPDGADRAAGRVPGFGVITNI
Bean	VISFKSALWFWMTAQSPKPSSHDVITSRWTPSSADVAARRLPGYGTVTNI
Potato	VISFKTALW--MTPQSPKPSCHDVIIGRWNPSSADRAANRLPGFGVITNI
Tobacco	VIS-KSALWFWMTPQSPKPSCHDVIIGRWQPSSADRAANRLPGFGVITNI
Tobacco	VISFKSALWFWMTPQSPKPSCHDVIIGRWQPSSADRAANRLPGFGVITNI
Coix	LDDYKTALXFIVNGEEAVPG LSAANA

	251 ... 300
Barley	IN****
Bean	INGGLECGRGQDSRVQDRIGFFKRYCDLLGVGYGNNLDCYSQTPFGNSLL
Potato	INGGLECGRGTDNRVQDRIGFYRRYCSILGVTPGDNLDCVNQRWFGNALL
Tobacco	INGGLECGRGTDSRVQDRIGFYRRYCSILGVSPGDNLDCGNQRSFGNGLL
Tobacco	INGGLECGRGTDSRVQDRIGFYRRYCSILGVSPGDNLDCGNQRSFGNGLL
Coix	VSYYRQYCQQLGVDPGPNL

	301
Bean	LSDLVTSQ*****
Potato	VDTL*****
Tobacco	VDTM*****
Tobacco	VDTM*****

Figure 4.2 Chou-Fasmann plot of the partial peptide sequence of the *Coix* α-amylase inhibitor.

A computer aided analytical method was used when designing the α–amylase inhibitor peptide antigen. This allowed some insight into the 3-dimensional structures coded in the primary sequence. The hydrophilicity predictions was carried out according to Kyte and Doolittle (1982) and the secondary structure according to Chou-Fasmann. The complete partial peptide sequence was analysed.



PLOTSTRUCTURE of: donna.gcg ck: 1542

P1;Donna - Donna's sequence - Coix

Chou-Fasman Prediction
November 25, 1991 09:28

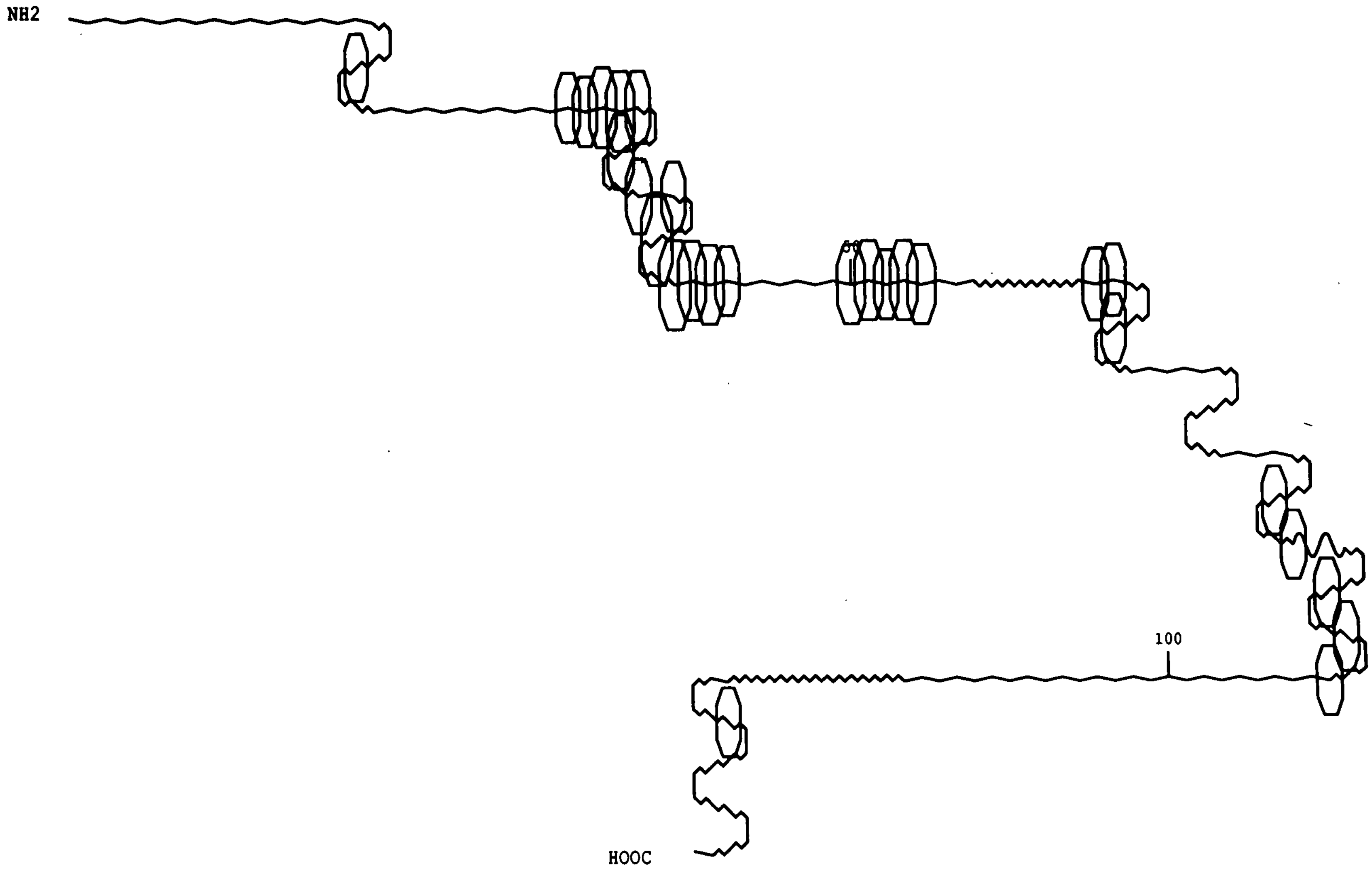


Figure 4.3 Table of antigenic indexes for α-amylase inhibitor peptide sequence

The partial peptide sequence of the *Coix* α-amylase inhibitor was analysed by Chou-Fasmann and antigenic indexes calculated for each amino acid. A stretch of amino acids with the highest antigenic indexes areshown in this table.

Pos	Position
AA	Amino acids
Glycos	Glycosylation site
Hyphil	Hydrophilicity (Kyte and Doolittle) averaged over a window of 7
SurfPr	Surface probability according to Emini
FlexPr	Chain flexibility according to Karplus Schulz
CF prediction	Secondary structure according to Chou-Fasman
GOR prediction	Secondary structure according to Garnier-Osguthorpe-Robson
AI Ind	Antigenic index

Pos	AA	Glycos	Hyphil	SurfPr	FlexPr	CF pred	GORPred	AI Ind
1	N	.	1.625	1.533	1.000	.	T	1.300
2	N	.	0.800	0.643	1.000	.	T	1.150
3	A	.	1.250	0.840	1.000	T	T	1.550
4	Y	.	1.300	0.808	1.000	T	T	1.550
5	C	.	0.914	0.673	0.967	.	T	1.000
6	D	.	0.971	1.333	1.013	.	T	1.300
7	P	.	1.329	1.227	1.053	T	T	1.700
8	S	.	1.643	3.965	1.093	T	T	1.700
9	K	.	2.557	4.748	1.108	t	T	1.500
10	T	.	2.286	4.748	1.108	T	T	1.500
11	Q	.	1.700	1.899	1.098	h	T	1.300
12	K	.	1.329	0.959	1.063	h	.	0.750
13	P	.	0.514	0.672	1.024	h	T	1.150
14	C	.	0.471	0.384	0.983	h	T	0.700
15	A	.	0.529	0.384	0.968	h	T	1.000
16	A	.	0.529	0.496	0.981	h	T	1.000
17	G	.	0.486	1.451	1.003	.	T	1.000
18	K	.	1.029	2.250	1.020	t	T	1.500
19	K	.	1.343	2.204	1.021	t	T	1.500
20	Y	.	2.243	4.363	1.007	.	T	1.300
21	Y	.	2.243	2.159	1.007	.	T	1.500
22	G	.	1.914	1.669	1.024	t	T	1.500
23	R	.	0.714	0.747	1.042	t	B	0.950
24	G	.	1.029	0.825	1.053	t	B	0.950
25	P	.	0.200	0.585	1.041	.	B	0.450
26	I	.	0.257	0.400	1.018	B	B	0.450
27	Q	.	0.114	0.650	0.998	B	B	0.300
28	I	.	0.243	0.659	0.992	B	B	0.300
29	S	.	0.514	1.511	0.990	B	B	0.750
30	N	.	1.343	1.367	0.985	B	T	1.150
31	Y	.	0.900	1.930	0.982	B	T	1.150
32	N	.	1.771	2.227	0.982	t	T	1.350
33	Y	.	1.400	1.399	O.994	t	T	1.350

Figure 4.4 The sequence of two complementary oligonucleotides encoding an α-amylase inhibitor peptide.

Using *E.coli* codon preference data two complementary 109mer oligonucleotides were designed to encode the 33 amino acid *Coix* peptide. The oligonucleotides were annealed to leave EcoRI and BamH1 overhangs to facilitate cloning into pGEX3X.

Coix α-amylase inhibitor peptide sequence

N N A Y C D P S K T Q K P C A A G K K Y Y G R G P I Q S N Y N Y

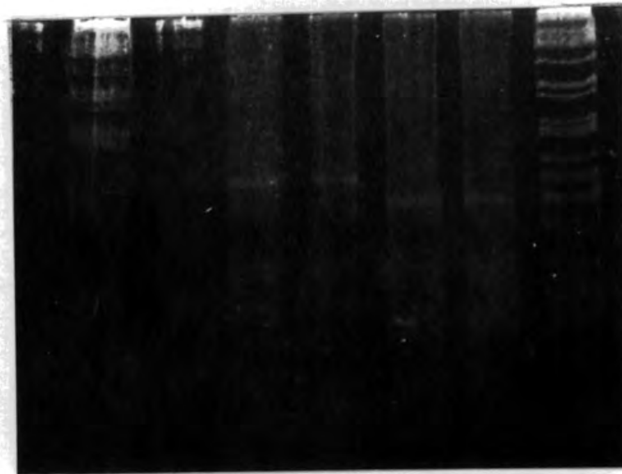
Oligonucleotides 462/463

```
  BamH1
5'  GATCCTGAACAACGCCTACTGCGATCCGAGCAAAACCCAG
    3'   GACTTGTTGCGGATGACGCTAGGCTCGTTTTGGGTC

AAACCGTGCGCCGCCGGCAAAAAATACTACGGCCGCGGCCCGA
TTTGGCACGCGGCGGCCGTTTTTTATGATGCCGGCGCCGGGCT

TTCAGATTAGCAACTACAACTACGTG   3'
AAGTCTAATCGTTGATGTTGATGCACTTAA   5'
                          EcoR1
```

Figure 4.5 DNA gel electrophoresis of oligonucleotides, 462 and 463 which encode for the *Coix* α-amylase inhibitor peptide.

The oligonucleotides were electrophoreised in a DNA acrylamide gel to check their yield and integrity, prior to annealing.

Lane 1	λPst I DNA size markers
Lane 2	Blank lane
Lane 3	Oligonucleotide 462, 396ng
Lane 4	Oligonucleotide 462, 1μg
Lane 5	Oligonucleotide 463, 512ng
Lane 6	Oligonucleotide 463, 1μg
Lane 7	λPst I DNA size markers


1
2
3
4
5
6
7
oligo 462
oligo 463

1Blue cells (section 2.9). Transformants were selected on LB Amp/Tet plates and 47 putative positives restreaked on LB Amp/Tet plates for screening by colony hybridisation.

Putative positive colonies were transferred onto nitrocellulose filters and screened with oligonucleotide 462 end labelled with $^{32}P\alpha$-dCTP (section 2.10.13.3), as described in section 2.10.14. 19 positive hybridisations were obtained from a total of 47 colonies.

Plasmid DNA was purified from one of the positive colonies (section 2.8.1). This plasmid was named pDF 5. 1ug of pDF 5 DNA and pGEX3X DNA was double digested with Bam HI and PstI (section 2.10.3), and electrophoresed alongside λPst I DNA markers on a 2% agarose gel (2.10.10). pGEX3X should release a fragment of 967bp on digestion with BamH1 and EcoRI, whereas pDF 5 should release a fragment of 1076 bp. A size shift of the inserts released confirmed that subcloning of the 109 bp fragment had been successful (results not presented).

To establish that the integrity of the oligonucleotides designed to encode the peptide had been maintained, and that the insert DNA was cloned into pGEX3X in frame for expression, pDF 5 DNA was manually sequenced (2.27.1). Due to the probable formation of hairpin structures in GST sequences near the cloning sites, a sequencing primer complementary with the extreme 3' end of the GST gene was used for sequencing. The insert sequence was intact and in frame with the GST coding sequence for expression (results not presented).

4.3.2 pDF 5 expression studies.

To monitor expression of the GST-fusion peptide, expression studies were carried out on the vector pDF 5. DNA fragments cloned into pGEX3X are expressed under the control of the *E.coli tac* promoter which is inducible following exposure to IPTG.

XL-1Blue transformed with pDF 5 (section 4.3.1), induced and non induced were analysed for expression. Similiar cultures inoculated with *E.coli* XL-1Blue alone and XL-1Blue transformed with pGEX3X, induced and non induced, were also examined for expression. Crude total protein extracts were prepared from these six samples and analysed by SDS-PAGE (section 2.15.1.) Figure 4.6. These results suggested the following,

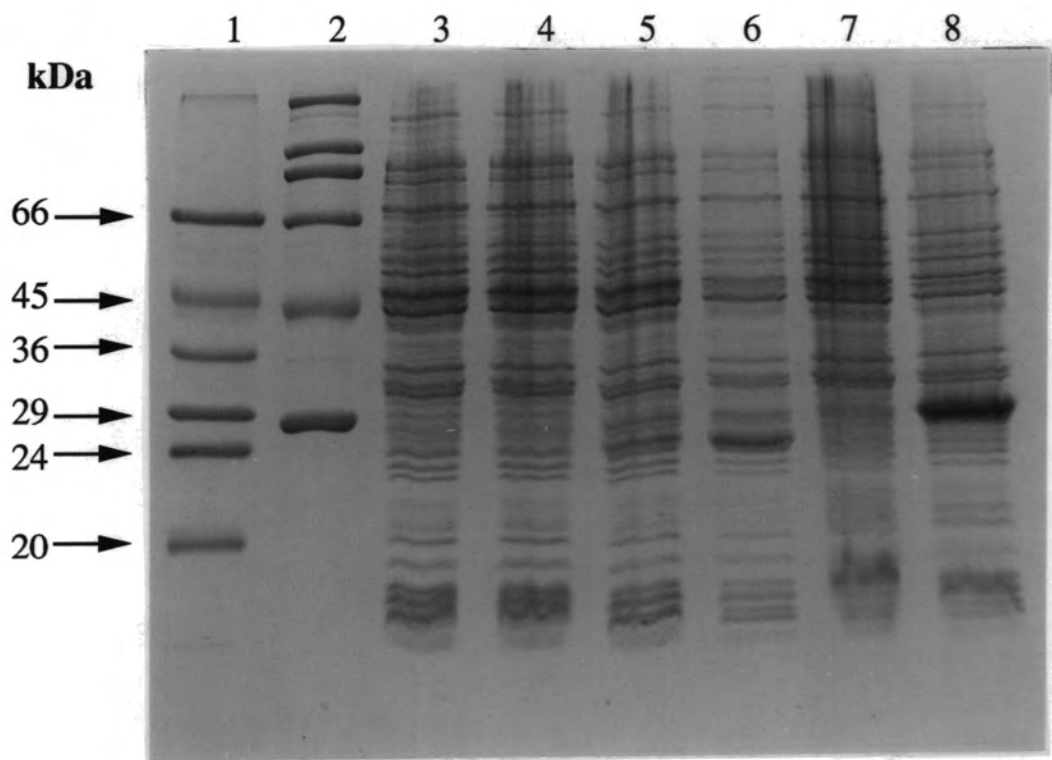
(i) Glutathione-S-transferase is not being expressed in induced or non induced *E.coli* XL1-Blue cells as shown lanes 3 and 4.

(ii) GST (approximate molecular weight 26,000Da), is being expressed, only when induced with IPTG, in XLI-Blue cells harboring pGEX3X, as shown in lane 6.

Figure 4.6 SDS-PAGE analysis of crude protein extracts of *E.coli* XL1-Blue expressing GST-α-amylase inhibitor fusion protein

Protein were isolated from liquid cultures containing the following innoculants (2.17) and then analysed by SDS-PAGE (section 2.15.1). Proteins were visualised by staining with coomassies blue (section 2.15.3.1)

Lane 1	Low molecular weight standards
Lane 2	High molecular weight standards
Lane 3	*E.coli* XL1-Blue, non induced
Lane 4	*E.coli* XL1-Blue, induced
Lane 5	*E.coli* XL1-Blue, transformed with pGEX3X, non induced
Lane 6	*E.coli* XL1-Blue, transformed with pGEX3X, induced
Lane 7	*E.coli* XL1-Blue, transformed with pDF5, non induced
Lane 8	*E.coli* XL1-Blue, transformed with pDF5, induced



1
2
3
4
5
6
7
8
kDa
66
45
36
29
24
20

(iii) The GST-fusion peptide, approximate molecular weight 29,500Da, is being expressed following induction by IPTG. The peptide encoded by the 109bp insert has a M*r* of approximately 3,630Da, lane 8.

4.4 Isolation of the GST-fusion peptide

Isolation of the GST-fusion peptide from the total *E.coli* proteins was necessary to facilitate the production of α-amylase inhibitor antiserum.

GST-fusion protein was purified from an IPTG induced 500ml liquid culture inoculated with *E.coli* XL1-Blue harbouring pDF 5, according to the method outlined in section 2.17. 8.9mg of fusion protein (following Bradford assay, section 2.15.5)) was isolated from the 500ml culture. Aliquots of the isolated proteins were electrophoresed by SDS-PAGE (2.15.1). For comparison, samples containing crude extracts of *E.coli* with induced GST and GST-fusion proteins and low and high molecular weight molecular markers were electrophoresed as described above, Figure 4.7. The GST-fusion peptide isolation procedure was succesful with minimal contamination with of non fusion protein (lanes 4, 5 and 6).

A Western blot was carried out to verify that the protein isolated was a GST-fusion protein. GST and the GST-fusion protein were probed with anti-GST primary antibody (a gift of Dr. Tommey, Durham University) Figure 4.8. The fusion protein isolated was immunopositive with the GST antiserum, therefore confirming it as a GST-fusion protein.

4.5.1 Production of GST-α-amylase inhibitor polyclonal antibodies.

The immunization procedure is described in detail in section 2.23.

Western blots of crude total protein extracts from leaves and seeds of *Coix* probed with GST antibody had resulted in no cross reaction with any of the seed and leaf proteins (results not presented). Therefore, it was concluded that antibodies raised aginst the carrier GST protein would not result in false positives when immunoscreening the λgt11 expression library. Therefore removal of the carrier protein was not required.

Briefly, immunization schedule undertaken for raising antibodies against the GST-fusion protein consisted of primary exposure to the antigen in complete adjuvant, followed by 3 boosts in incomplete adjuvant prior to terminal bleed. Tail bleeds were carried out on each animal 10 days after the injection, to allow monitoring of the antibody response (section 2.23).

Figure 4.7 SDS-PAGE analysis of purified GST-fusion protein

The GST-α-amylase inhibitor fusion protein was isolated from large scale liquid culture (section 2.17) Proteins isolated were analysed by SDS-PAGE and stained with coomassie blue (section 2.15.1 and 2.15.3.1.).

Lane 1	Crude total protein extracts from *E.coli* XL1-Blue expressing GST
Lane 2	Purified GST
Lane 3	Crude total protein extracts from *E.coli* XL1-Blue expressing GST-α amylase inhibitor fusion protein
Lane 4	1.58μg of purified GST-fusion protein
Lane 5	4.74μg of purified GST-fusion protein
Lane 6	7.9μg of purified GST-fusion protein
Lane 7	Low molecular weight standards
Lane 8	High molecular weight standards

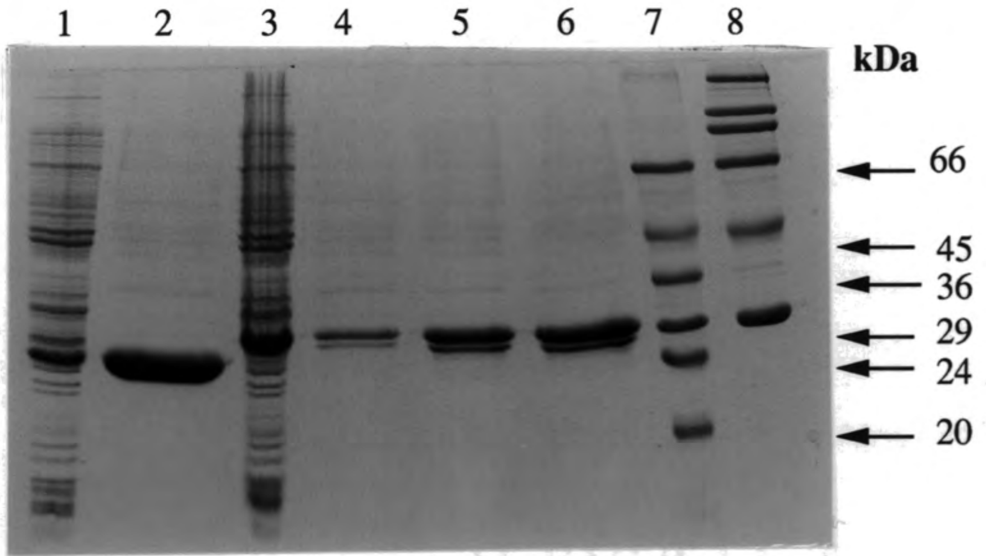
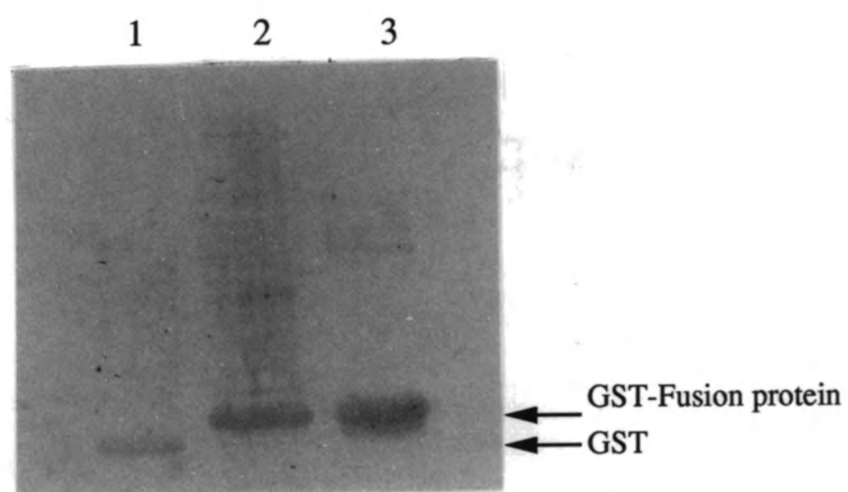

1
2
3
4
5
6
7
8
kDa
66
45
36
29
24
20

Figure 4.8 Western blot of GST-α-amylase inhibitor fusion protein probed with anti-GST serum

Proteins were seperated by SDS-PAGE (section 2.25.1), transferred to nitrocellulose, and then probed with anti-GST antibodies using alkaline phosphatase conjugated secondary antibody (section 2.15.2).
The protein loading for each lane are described below.

Lane 1	Purified GST
Lane 2	Crude protein extract of *E.coli* XL1-Blue transformed with pDF5 expressing GST-α-amylase inhibitor fusion protein
Lane 3	Purified GST-α-amylase inhibitor fusion protein


1
2
3
GST-Fusion protein
GST

Primary and secondary tail bleeds were obtained 10 days after the first and second boosts. Bleeds from all six mice were combined and a 1/500 dilution used to probe GST-fusion and GST proteins (2.15.1). This was to monitor the titre of the antibodies being raised against the GST-fusion antigen.

1/500 dilutions from primary bleeds gave immunopositive reactions with both GST and GST-fusion proteins as shown in Figure 4.9. An immune response elicited by the antigen was occuring in mice. The antibody was specifically immunopositive GST-fusion protein in protein extracts from induced *E.coli* XL-IBlue expressing pUCDF 5. There was no cross reaction against other *E.coli* proteins.

4.5.2 Characterisation of terminal bleed from immune mice.

3 days after the 3rd boost injection the mice were terminally bled to give 7 samples of serum, 1 of them being non immune (section 2.23). All samples were stored separately so that their respective titres could be determined because the immune response of individual mice can vary considerably within a population.

7 strips of nitrocellulose (X2) onto which the GST-fusion peptide had been blotted were probed with 1/500 and 1/1000 dilutions of the 7 terminal bleeds in order to check the titre of each. All 6 immune terminal bleeds gave strong immunopositive reactions with 1/500 dilutions, Figure 4.10. There is some variation between the titres obtained from samples 1-6. Samples 2 and 3 gave the highest titres and so were used for all subsequent assays. Only bleeds 2 and 3 were faintly immunopositive with 1/1000 dilutions, (results not presented). An extremely faint positive reaction was obtained with 1/500 dilution of the non immune serum whereas 1/1000 dilution gave no detectable reaction.

4.5.3 Western blots of total protein extracts from *Coix* seeds probed with GST-fusion protein antiserum

Prior to immunoscreening of the seed λgt11 expression library with the GST-α-amylase inhibitor antiserum, it was crucial to ascertain whether the immunoserum could detect the α-amylase inhibitor protein in total protein extracts from *Coix* seeds. It was also important that the antibodies did not crossreact with other seed proteins as this would generate false positives during the library immunoscreening.

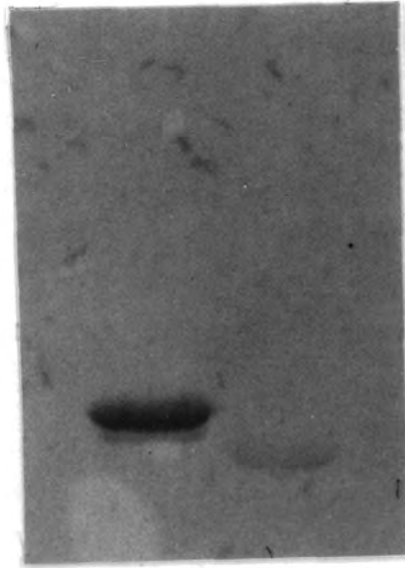
Coix seeds were harvested at several developmental stages (section 2.7), for example; Yellow, 4, 7, 10, 14, 18, 24 and 28 days after flowering (d.a.f.) and whole cell extracts prepared from each (section 2.16). The total protein concentration was estimated for each by

Figure 4.9 Western blot of GST-α-amylase inhibitor fusion protein, and GST, probed with GST-α-amylase inhibitor serum from primary bleed.

The proteins were seperated by SDS-PAGE (2.15.1) and transferred to nitrocellulose prior to probing with the combined immunoserum from six primary tail bleeds from mice (2.15.2). The mice had previously been injected with GST-α-amylase inhibitor fusion protein, with the aim of raising polyclonal antibodies against this protein (section 2.23). Alkaline phosphatase conjugated secondary antibody was used as the secondary antibody.

Lane 1 GST-α–amylase inhibitor fusion protein.

Lane 2 GST protein


1
2
GST-fusion protein
GST

Bradford assays (2.15.5), and approximately equal protein loadings from each stage analysed by Shäegger gel electrophoresis, Figure 4.11. This was to check the protein isolation loadings. Crude protein extracts from the 8 stages of *Coix* seed development were analysed by SDS-PAGE and transferred onto nitrocellulose (section 2.15.1). The proteins were probed with a 1/500 dilution of GST-fusion peptide antiserum (2.15.2) and immunopossitive proteins detected using alkaline phosphatase-conjugated secondary antibody. A very faint immunopositive band was obtained corresponding to a M*r* of approximately 26,000 Da, in 24 and 28 d.a.f. protein extracts. However this band was too faint to photograph (results not presented).

4.6. Detection of immunopositive proteins using an I^{125} detection system

For further analysis of these immunopositive proteins, a more sensitive detection system was utilised, this was an I^{125} labelled anti-IgG. Due to the fact that the α–amylase inhibitor immunserum was raised in mice and the I^{125} conjugate available was anti-rabbit, a sandwich reaction with anti-mice rabbit conjugate was performed. The probed filters were exposed to X-ray film and developed at -70^{0}C (section 2.11).

4.6.1 Western blots of total protein extracts from *Coix* seeds probed with GST-fusion protein antiserum

The serum crossreacted with several proteins in all the protein samples apart from the yellow seed preparation, Figure 4.12. This lack of immunopositive reaction in the yellow seed sample was probably due to the insufficient protein loading in the sample. An immunopositive protein with M*r* approximately 26,000Da was detected in the 24 and 28 daf seed extracts. The serum was immunopositive against the GST-α-amylase inhibitor protein positive control.

4.7 Affinity purification of GST-α-amylase inhibitor protein antiserum.

4.7.1 Affinity purification against GST-α-amylase inhibitor protein.

To reduce the non specific cross reaction of the antiserum with *Coix* seed proteins, affinity purification against the GST-α–amylase inhibitor fusion protein was attempted (section 2.24). Only antibodies raised specifically against the GST-α–amylase inhibitor fusion protein should remain in the antibody preparation.

300μg of GST-α-amylase inhibitor protein was separated on a preparative 12% gel by SDS-PAGE (section 2.15.1). The protein was transferred onto nitrocellulose overnight and the proteins visualised following staining by Ponceau S (section 2.15.2). A horizontal strip of

Figure 4.11 Shägger gel electrophoresis of crude protein extracts from seeds of *Coix* at several developmental stages.

Coix seeds were harvested at several stages, up to 28 days after flowering (section 2.7) and whole cell extracts prepared (section 2.16). The protein extracts were anaylased by Shägger gel electrophoreis as shown below.

Lane 1	Protein molecular weight markers
Lane 2	Protein extracts from seeds 28 daf
Lane 3	Protein extracts from seeds 24 daf
Lane 4	Protein extracts from seeds 18 daf
Lane 5	Protein extracts from seeds 14 daf
Lane 6	Overflow from lane 5
Lane 7	Protein extracts from seeds 10 daf
Lane 8	Protein extracts from seeds 7 daf
Lane 9	Protein extracts from seeds 4 daf
Lane 10	Protein extracts from yellow seeds

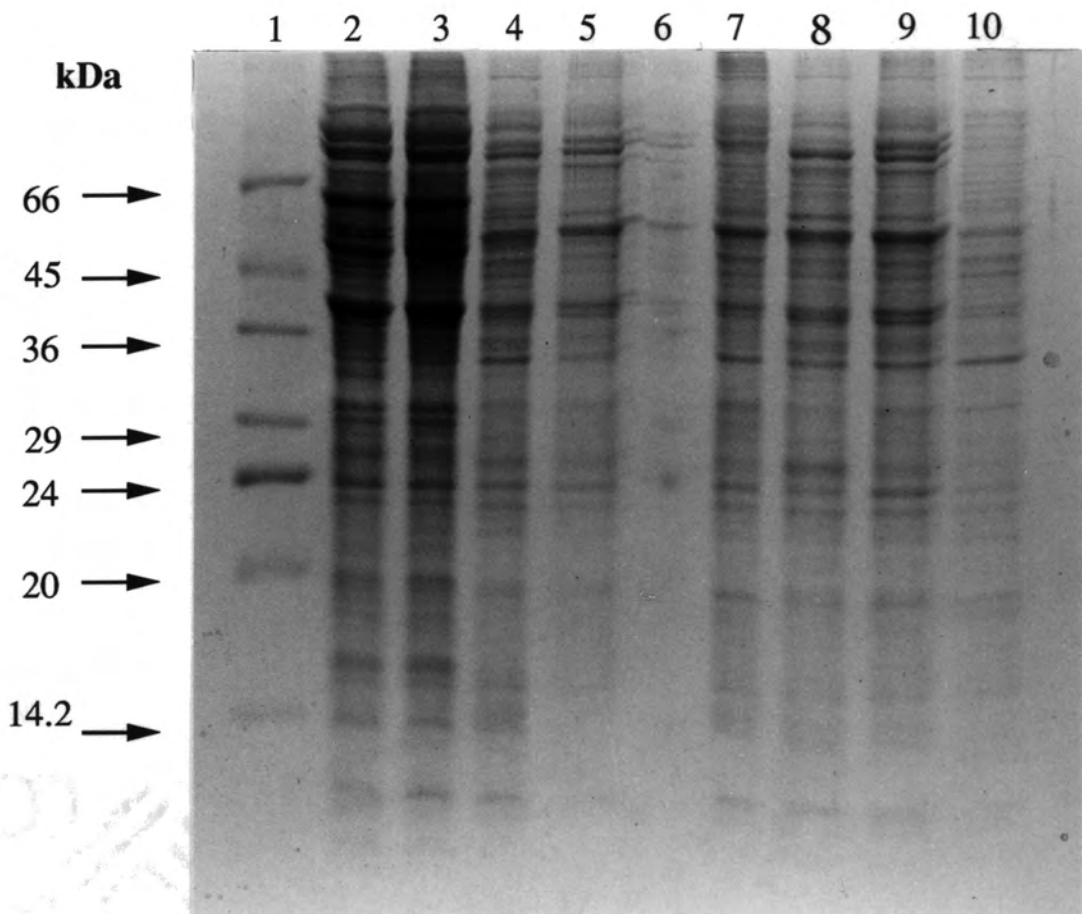
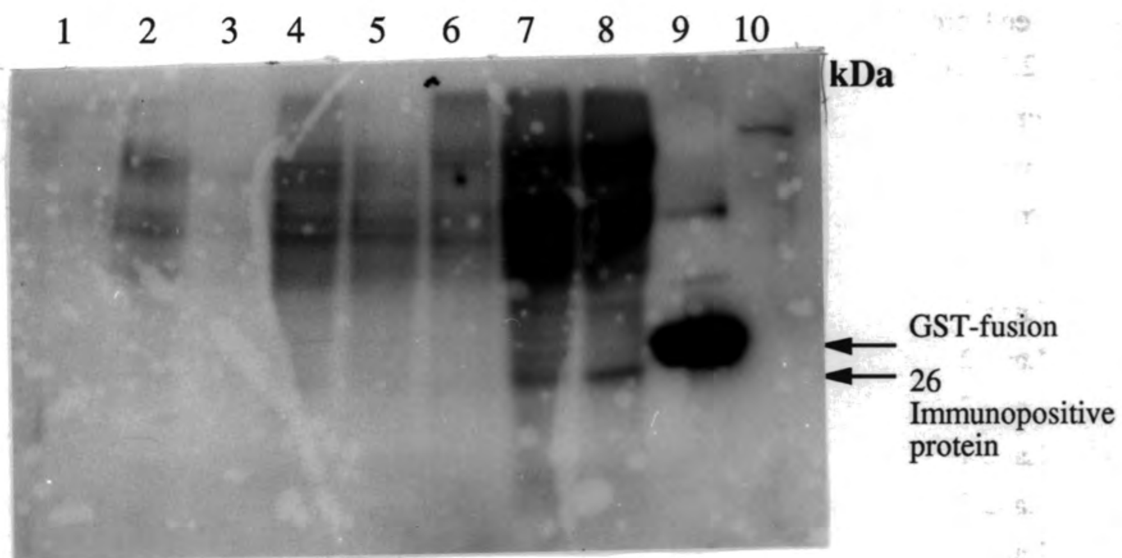

1
2
3
4
5
6
7
8
9
10
kDa
66
45
36
29
24
20
14.2

Figure 4.12 Western blot of crude protein extracts from *Coix* seeds probed with crude GST-α–amylase inhibitor immunoserum.

Seed proteins were separated by SDS-PAGE (2.15.1) and transferred to nitrocellulose (2.15.2). *Coix* seed proteins were probed with immunoserum raised against the GST–α-amylase inhibitor using an I^{125} conjugated IgG as secondary antibody. The blot was exposed overnight prior to development (section 2.11).
Protein loadings are described below;

Lane 1	Protein extracts from Y seeds
Lane 2	Protein extracts from 4 daf seeds
Lane 3	Protein extracts from 7 daf seeds
Lane 4	Protein extracts from 10 daf seeds
Lane 5	Protein extracts from 14 daf seeds
Lane 6	Protein extracts from 18 daf seeds
Lane 7	Protein extracts from 24 daf seeds
Lane 8	Protein extracts from 28 daf seeds
Lane 9	GST-α-amylase inhibitor protein
Lane 10	High molecular weight markers


1
2
3
4
5
6
7
8
9
10
kDa
GST-fusion
26
Immunopositive
protein

nitrocellulose containing only the GST-α-amylase inhibitor protein was excised and used to affinity purify the antibody as described in section 2.24.

A 1/45 dilution (this was equivalent to a 1/500 dilution of the crude antisera) of the affinity purified antibody was used to probe the seed protein, Figure 4.13. The serum remained immunopositive against the 26,000Da protein with the intensity and number of non specific crossreactions being reduced. The serum still crossreacted non specifically with several proteins in the 24 and 28daf seed protein samples.

Following an overnight exposure, the 26,000Da protein was shown to be immunopositive in the 28, 24, 10 and 4 d.a.f. seed protein preparations. This Western blot is shown in Figure 4.13A. Following exposure for 4 days, the 26,000Da protein was immunopositive in all protein samples apart from the yellow seed stage Figure 4.13B.

4.7.2 Affinity purification against GST protein

To determine whether the 26,000Da protein was as a result of anti-GST or anti-α-amylase inhibitor antibodies, the following affinity purification procedure was carried out. A strip of nitrocellulose onto which GST protein alone had been blotted was coincubated with the GST-α–amylase inhibitor immunoserum. GST antibodies in the imunoserum should bind to GST on the nitrocellulose, thus removing any anti-GST antibodies from the antiserum (section 2.24). This remaining serum should only contain antibodies against the α–amylase inhibitor peptide moiety of the GST-α-amylase inhibitor fusion peptide.

The affinity purified antibodies were used to probe *Coix* seed proteins (2.15.5) using the I^{125} detection system. Although several non specific immunopossitive proteins were visualised, the 26,000Da band was no longer immunopositive following a four day exposure, Figure 4.14. However the immunoserum was still immunopositive against the GST-α-amylase inhibitor protein.

4.8.1 Factor Xa cleavage of GST-α–amylase inhibitor fusion protein

To determine whether the antibodies raised against the GST-α-amylase inhibitor peptide contained antibodies specifically against the α-amylase inhibitor peptide, cleavage of the fusion protein into GST and α–amylase inhibitor fractions was attempted. The strategy was to seperate the cleavage products by electrophoresis, transfer the proteins to nitrocellulose and probe the proteins with the GST-fusion protein antibodies. The antiserum should be immunopositive against both proteins.

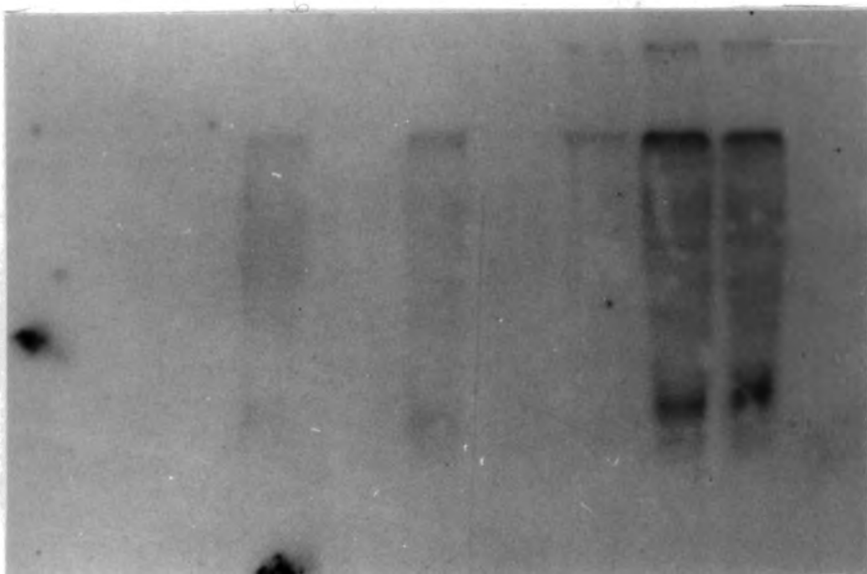
Figure 4.13 Western blot of crude protein extracts from *Coix* seeds probed with affinity purified GST-α–amylase inhibitor antibodies.

The *Coix* seed proteins were probed with immunoserum, previously affinity purified with GST-α-amylase inhibitor fusion protein (section 2.24), using a I^{125} conjugated IgG detection method.

The blot was exposed for a) overnight and b) 4 days (section 2.11) to visualise immunopositive proteins.

The protein loadings are described below;

Lane 1	High molecular weight markers
Lane 2	Low molecular weight markers
Lane 3	Protein extracts from Yellow seeds
Lane 4	Protein extracts from 4 daf seeds
Lane 5	Protein extracts from 7 daf seeds
Lane 6	Protein extracts from 10 daf seeds
Lane 7	Protein extracts from 14 daf seeds
Lane 8	Protein extracts from 18 daf seeds
Lane 9	Protein extracts from 24 daf seeds
Lane 10	Protein extracts from 28 daf seeds


a
1
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4
5
6
7
8
9
10
26 kDa
Immunopositive
protein

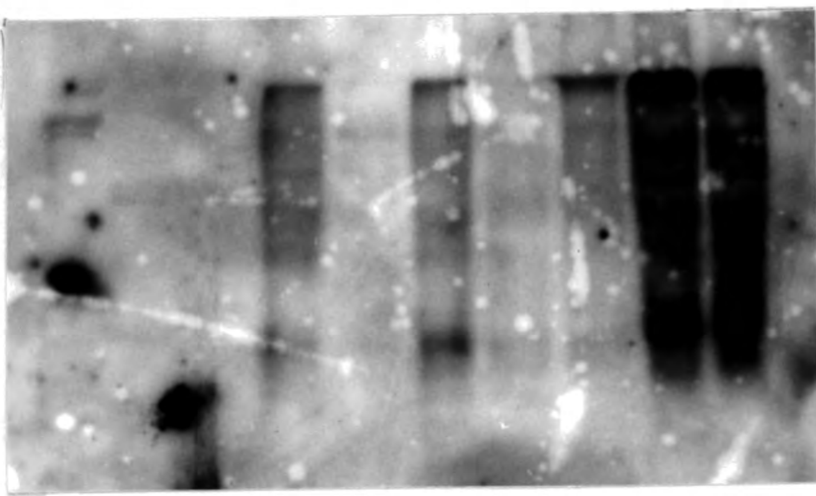
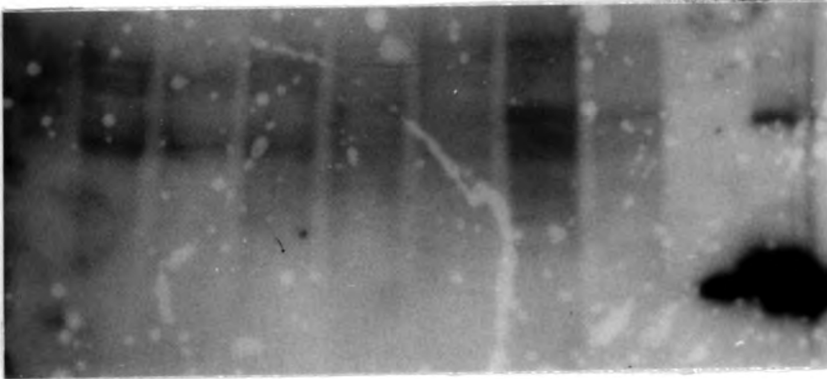

b
1
2
3
4
5
6
7
8
9
10
26 kDa
Immunopositive
protein

Figure 4.14 Western blot of crude protein extracts from *Coix* seeds probed with affinity purified putative α-amylase inhibitor antibodies.

The seed proteins were probed with immunoserum initially raised against the GST-α-amylase inhibitor protein (section (2.23), susequently affinity purified to remove antibodies against GST (section 2.24). An I^{125} conjugated IgG detection method was used for detection of immunopositive proteins.
The blot was exposed for 4 days prior to development

The protein loadings are as follows;

Lane 1	Protein extracts from Yellow seeds
Lane 2	Protein extracts from 4 daf seeds
Lane 3	Protein extracts from 7 daf seeds
Lane 4	Protein extracts from 10 daf seeds
Lane 5	Protein extracts from 14 daf seeds
Lane 6	Protein extracts from 18 daf seeds
Lane 7	Protein extracts from 24 daf seeds
Lane 8	Protein extracts from 28 daf seeds
Lane 9	High molecular weight markers
Lane 10	GST–α-amylase inhibitor fusion protein


1 2 3 4 5 6 7 8 9 10
GST-Fusion

pGEX3X encodes a protease cleavage site so that cloned protein can be released from the GST moiety. The recognition sequences for the blood coagulation factor Xa (Nagai and Thorgerson, 1984) lies between GST and the amino acids encoded by the polylinker sequence.

Two methods were used in an attempt to cleave the fusion protein. Firstly cleavage was attempted on the pure fusion protein in solution (Smith and Johnson, 1988) and secondly cleavage was attempted while the fusion was still bound to the glutathione column (Gearing *et al.,* 1989) as described in section 2.18. The cleavage products were analysed by SDS-PAGE (section 2.15.1), and Shägger gel electrophoresis (16% running gel) as described in Shägger and von Jagow, 1987). Shägger gel electrophoresis was carried out because it is more appropriate for resolving smaller molecular weight proteins such as the α-amylase inhibitor peptide, which is approximately 3,630Da in size. Proteins were visualised by coomassie blue staining (section 2.15.3.10), followed by staining of the SDS gel by silver staining (section 2.15.3.2). The gels were silver stained because even under optimum cleavage conditions, coomassie blue may not have been sensitive enough for detection of the α-amylase inhibitor cleaved moiety.

Unfortunately no cleavage was detected following either of the methods, (results not presented). It was assumed that this was because the factor Xa could not access its cleavage site. Therefore an attempt was made to denature the protein with the aim of opening up the factor Xa cleavage site.

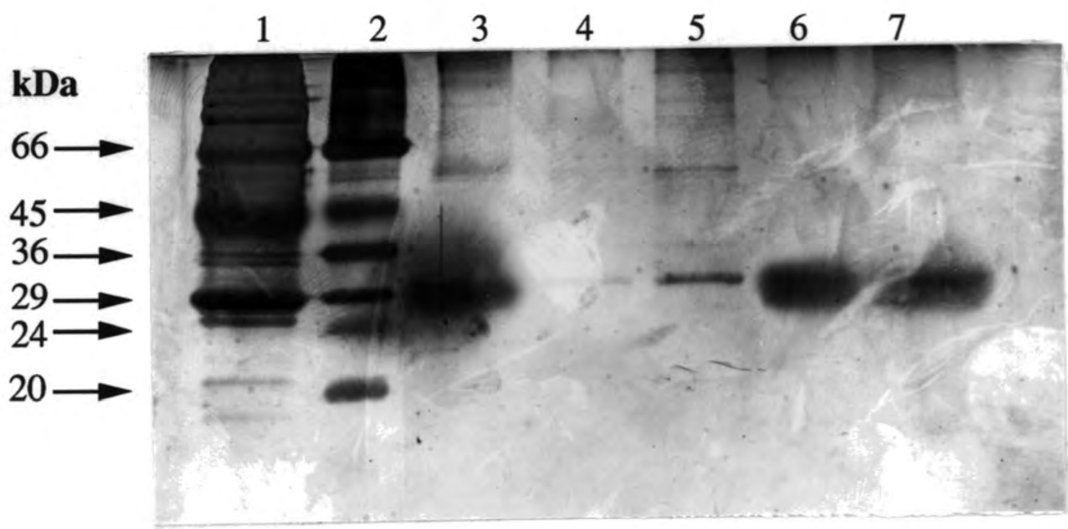
4.8.2 Factor Xa cleavage of GST-α-amylase inhibitor protein following denaturation with SDS

In an attempt to partially unfold the protein, the cleavage reaction was carried out in cleavage buffer containing 0.01% and 0.001% SDS (final concentration). Cleavage products were analysed as described in section 4.8.1, by SDS and Shägger gel electrophoresis. The resulting protein gels are shown in Figures 4.15 and 4.16. The addition of SDS did not result in cleavage of the GST-α-amylase inhibitor, following analysis of products following cleavage by SDS-PAGE and gel staining.

Figure 4.15 SDS-PAGE analysis of products following attempted cleavage of GST-α-amylase inhibitor fusion protein with Factor Xa.

The GST-α-amylase inhibitor fusion protein was incubated with Factor Xa, in an attempt to cleave the fusion protein into GST and α-amylase inhibitor fractions (section 2.18). Following cleavage in the presence of SDS, the products were analysed be SDS-PAGE, as shown below (section 2.15.1).

Lane 1	High molecular weight protein markers
Lane 2	Low molecular weight protein markers
Lane 3	GST-α-amylase inhibitor fusion protein prior to cleavage
Lane 4	Cleavage products after separation with centricon column (cleavage in the presence of 0.01% SDS).
Lane 5	Cleavage products after separation with centricon column (cleavage in the presence of 0.001% SDS).
Lane 6	Cleavage products in the presence of 0.01% SDS
Lane 7	Cleavage in the presence of 0.001% SDS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kDa
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36
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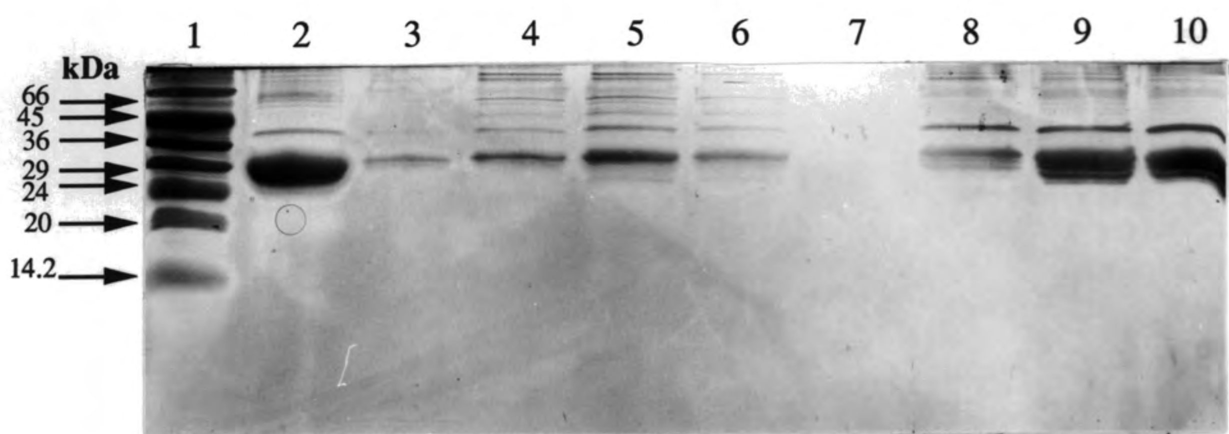
24
20

Figure 4.16 Schaegger gel electrophoresis following attempted cleavage of GST-α-amylase inhibitor fusion protein with Factor Xa.

The GST-α-amylase inhibitor fusion protein was incubated with Factor Xa, in an attempt to cleave the fusion protein into GST and α-amylase inhibitor fractions (section 2.18). Following cleavage, the products were analysed be Shaegger gel electrophoresis, as shown below (section 2.15.1).

Lane 1	Low molecular weight protein markers
Lane 2	GST-α-amylase inhibitor fusion peptide
Lane 3	5μl of products following the cleavage of the fusion protein with Factor Xa in 0.01% SDS
Lane 4	10μl of products following the cleavage of the fusion protein with Factor Xa in 0.01% SDS
Lane 5	15μl of products following the cleavage of the fusion protein with Factor Xa in 0.01% SDS
Lane 6	Overflow from lane 5
Lane 7	Blank
Lane 8	5μl of products following the cleavage of the fusion protein with Factor Xa in 0.001% SDS
Lane 9	10μl of products following the cleavage of the fusion protein with Factor Xa in 0.001% SDS
Lane 10	15μl of products following the cleavage of the fusion protein with Factor Xa in 0.001% SDS


1
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9
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kDa
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36
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14.2

4.9 DISCUSSION

4.9.1 The overall strategy.

An α–amylase inhibitor/glutathione-S-transferase peptide was isolated following expression in the bacterial expression vector pGEX3X. This fusion peptide was used to raise antibodies in mice, which once characterised were to be used to screen a λgt11 expression library to isolate the *Coix* α-amylase inhibitor/endochitinase cDNA. Initial characterisation of the antibody revealed that it did cross react with a protein M*r* 26,000Da (as estimated by SDS-PAGE). The estimated size of the α-amylase inhibitor under similiar assay conditions is 26,400Da. However, the antibody was very non specific and would have generated several false possitives following immunoscreening of the λgt11 cDNA library .

The antibodies were affinity purified, firstly against the fusion peptide with the aim of removing antibodies non specific to the fusion Secondly, to remove antibodies against GST theoretically leaving those antibodies specific to the α-amylase inhibitor moiety, in the immunoserum. Unfortunately the problem of whether or not antibodies had been raised against the α-amylase inhibitor peptide was undecided.

4.9.2 Choice of antigen.

The initial decision to raise the antibodies against a fusion peptide rather than the native α-amylase inhibitor was two fold. Firstly, the protein which had originally been purified by Ary *et al.* (1989) was no longer available and it would have been necessary to reisolate the protein from *Coix* seeds. Secondly, using a bacterial expression vector to synthesise an immunogen was a technique which had previously been succesful in Durham. With hindsight it may have been interesting to raise antibodies against both the native protein and the fusion peptide and to compare and contrast both methods as to their suitability for raising antibodies.

The immunoserum raised against the GST-α-amylase inhibitor peptide was native serum because in no case was the fusion protein denatured during the isolation procedure. At the time this was appropriate because the antibodies were to be used to screen a cDNA expression library, in which native proteins are expressed in *E.coli*. The fact that the immunoserum was used to detect denatured protein when carrying out Western blots may be significant.

It may have been appropriate to raise antisera against both native and denatured protein. Denaturing could have been carried out by SDS or heat treatment prior to injection of the

mice. Heating changes the structure of the proteins and as a result exposes more epitopes. Heating also causes protein antigens to aggregate and as aggregated antigens are more immunogenic, this may increase the antibody response. If in fact the major α-amylase inhibitor antigenic epitope predicted was "hidden" by the effect of being coupled to GST then denaturing the antigen may have been worthwhile.

4.9.3 Specificity of the GST-α-amylase inhibitor immunserum.

Initial characterisation of the immunoserum had revealed a positive reaction with a protein approximately M*r* 26,000 Da in seeds 24 and 28 daf. Initially this appeared to be a promising result because under denaturing conditions, the α-amylase inhibitor dissociates into 2 subunits M*r* 26,400. However there were many non specific cross reactions which would have made screening a cDNA library unrealistic, considering the number of false positives which may have been generated. Polyclonal antisera are frequently not monospecific since they often contain antibodies to contaminating antigens present in the original immunogen. Infact during purification of the fusion protein, several proteins had been coisolated. The majority of these were higher molecular weight proteins. Although these antigens may have been present in low concentration, i.e., less than 5%, they may be far more immunogenic than the antigen of interest. If this was the case, then they would constitute the majority of antibodies in the immunoserum, and the immunoserum may not give a strong immunopositive result against the 'real' antigen. Therefore an attempt was made to clean up the immunoserum by affinity purification.

4.9.4 The effect of affinity purification of the GST-α-amylase inhibitor immunserum.

The method used was a simple, efficient and rapid procedure for removing cross reacting or contaminating antibodies from a relatively crude antiserum in quantities large enough to screen a DNA library. Most other affinity purification methods involve direct coupling of the antigen to the matrix (e.g. cyanogen bromide activated Sepharose) without further purification. The resulting antibody preparation, therefore, would be a reflection of the purity of the antigen originally used as the immunogen, resulting in purification of contaminant antibodies. The method used here, has been used succesfully with antiserum to xylose isomerase to screen a plasmid expression library, and with an antiserum to bovine heavy neurofilament polypeptide for screening a λgt11 expression library (Robinson *et al.*, 1986).

Purification of the immunoserum with GST-α-amylase inhibitor to remove any antibodies non specific to this protein had resulted in a certain amount of purification of the immunoserum. The antisera was still cross reacting with several seed proteins, but to a lesser extent. Positive reactions were obtained against a 26,000Da protein in 24 and 28 daf protein

extracts, following probing of seed protein with affinity purified GST-α-amylase inhibitor antiserum (section 4.7.1) This is approximately the size expected for the *Coix* α-amylase inhibitior proteins subunit. Although these results looked encouraging, it was unclear whether the immunopositive protein was as a specific result of the α-amylase inhibitor antibodies.

When the anti-GST antibodies were removed by affinity purification, with the aim of leaving only α-amylase inhibitor antiserum, cross reaction against this 26,000Da was no longer obtained. Initially this implied that the 26,000Da immunopositive protein was a result of the interaction with the anti GST antibodies in the immunoserum.

However, this was unlikely for the following reasons. Prior to beginning this work, an initial consideration was that the GST antisera raised would cross react against the GST in plants. This would have made the identification of an immunopositive α-amylase inhibitor difficult because they are almost identical in *Mr*. To investigate this, *Coix* seed extracts were probed with antibodies raised against GST (gift Dr. A. Tommey). No cross reaction was obtained with any proteins, which was as expected because the GST which pGEX3X expresses is from the parasitic helminth, *Schistosoma japonicum*. This is only 40% similar to plant GST (personnel communication Dr. Tommey, University of Durham). Therefore it is unlikely that that the 26,000Da protein which cross reacts with GST-α-amylase inhibitor immunoserum is a plant GST.

An immunopositive reaction was obtained against the GST-α-amylase inhibitor protein, with immunoserum affinity purified to remove GST antibodies. Theoretically, all anti-GST antibodies should have been removed from the immunoserum by the affinity purification procedure. Realistically, anti-GST antibodies may still be present in the immunoserum and this may explain the immunopositive reaction with the fusion protein.

The immunopositive reaction with the 26,000Da protein could be as a result of cross reaction with some other uncharacterised antibodies which are contaminating the crude immunoserum preparation. The affinity purification procedure, to remove anti GST antibodies may have removed them from the immunoserum preparation, thus eliminating the positive reaction with the 26,000Da protein.

4.9.5 Factor Xa cleavage of the GST-α-amylase inhibitor protein.

An alternative approach to detemine whether antibodies had actually been raised against the the α–amylase inhibitor component of the fusion protein was to cleave the GST-α-amylase inhibitor with Factor Xa to release the α-amylase inhibitor peptide. The GST and α-amylase

inhibitor parts were to be separated by SDS-PAGE and then probed with the antiserum. It was hoped that the serum would crossreact with both the GST and the α-amylase inhibitor portions, thus concluding that α-amylase inhibitor antibodies were present in the antisera. Unfortunately, several attempts at cleaving the fusion protein failed.

Reports from the Sainsbury labs (personnel communcation Dr. Cummins, John Innes Institute) suggest that problems occur with peptides smaller than 5kD, when using fusion peptides as immunogens and when attempting to cleave with Factor Xa. Its possible that increasing the length of the peptide would have improved both of these features but a lack of contiguous amino acid sequence prevented this.

4.9.6 The antigenicity of the GST-fusion protein.

This brings in the question of the importance of peptide size and the nature of the carrier molecule. GST has been used succesfully as a carrier for numerous experiments and it was not forseen as being a problem. The nature of the carrier affects the local environment of the peptide molecule and possibly the folding of the peptide. Therefore once attached to the GST moiety, the α-amylase inhibitor peptide may not have folded as predicted by the Chou-Fasman plot. It is possible that the major antigenic sites predicted may not have been formed or if they were, did not form on the surface of the protein. It is conceivable that this situation occured with the GST-α-amylase inhibitor resulting in loss of antigenicity of the α–amylase inhibitor peptide.

The argument for this may be strengthened by the fact that attempts at cleaving the fusion peptide with factor Xa failed. One reason for this may be the inaccesibility of Factor Xa to access its cleavage site which lies between the GST moiety and the α-amylase inhibitor peptide, implying that the protein is misfolded. If cleavage of the fusion peptide had succeeded, it may have been linked to an alternative carrier molecule such as keyhole limpet haemocyanin to use as an immunogen.

4.9.7 Using fusion proteins as a method for producing antibodies.

Reports of succesful application of these antigenic determinant methods to obtain anti-protein antisera are numerous. However, there are many examples where the experiment failed. Hopp, (1984) was very succesful in raising antisera to hepatitis B virus using a synthetic peptide comprising the most hydrophilic site on the surface antigen. In a similiar study, another group reported negligible titres, using this segment (Lerner *et al.*, 1981). The only differences were that Hopp used a slightly longer peptide and the carrier, dipalmityl lysine, whereas Lerner *et al.* (1981) used a keyhole limpet hemocyanin conjugate attached to

a shorter peptide. Another example, yielded a totally opposite situation concerning the most hydrophilic segment of interleukin 2. Altman *et al.* (1984) reported obtaining very useful antisera against native interleukin 2, whilst Hopp, (1984) attempts at using the same peptide on the dipalmityl lysyl carrier were unsuccesful.

4.9.8 The *Coix* α-amylase inhibitor.

If antibodies against the α-amylase inhibitor had been produced then this raises the question of why the antibodies were not immunopositive against the α-amylase inhibitor in *Coix* seeds. With hindsight titration of the GST-fusion protein could have been carried out to estimate the minimum amount of protein which was required for an immunopossitive reaction. One possibility is that the concentration of α-amylase inhibitor present in the seed protein preparations was too small to be detected.

The assay for detection of the α–amylase inhibitor antibodies in the immunoserum was to probe crude protein extracts from *Coix* seeds at several stages of development with this immunoserum. Although no definitive test was carried out concerning whether the α–amylase inhibitor was present in these extracts, we can be extremely confident that it was. A sufficient range of stages were obtained in which the α-amylase inhibitor is expected to be synthesised. For example, in wheat, synthesis of the inhibitor starts about 8 days after fetilisation and rapidly increases with maturation up to a maximum reached at the full maturity (Pace *et al.*, 1978). Similiarly, an α-amylase inhibitor from *Phaseolus vulgaris* is synthesised during the same time as the main storage proteins which is 17 days after pollination. The amount of inhibitor increases until it reaches a plateau approximately 27dap (Moreno and Chrispeels, 1989).

CHAPTER 5

ISOLATION OF THE *COIX* α-AMYLASE INHIBITOR GENE USING THE POLYMERASE CHAIN REACTION.

5.1 Introduction.

Until recently, the most commonly used procedures in gene isolation required the establishment of a cDNA library from tissue or cellular RNAs. The gene of interest was then identified by screening the library with antibody (Young and Davis, 1983) or DNA probes (Suggs *et al.*, 1981). This approach has been very successful in cloning a great number of cDNAs which were then used to screen genomic libraries to isolate the genes. However, a new and faster approach to isolate new genes has been developed, namely the polymerase chain reaction (PCR).

The polymerase chain reaction (PCR) is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. The PCR method was designed by Mullis and colleagues at the Cetus Corporation (Mullis and Faloona, 1987), although the principle had been described in detail by and Panet and Khorana (1974).

The strategy employed during my work, was to use two oligonucleotide primers which were designed to hybridise to opposite strands and flank the region of the α-amylase inhibitor gene in the template DNA. The primers were oriented with their 3' ends pointing towards each other to allow amplification of the DNA sequence in between them.

Using PCR the α-amylase inhibitor gene could be amplified from the target *Coix lachryma jobi* cDNA and/or genomic DNA. The amplified product could then be used as a homologous hybridisation probe allowing high stringency screening of gene libraries to isolate full length cDNA or genomic clones.

5.1.1 The polymerase chain reaction conditions.

PCR amplification is performed by incubating the samples in a cycle of 3 temperatures corresponding to 3 steps, denaturation, annealing and extension. In a typical reaction, the double stranded DNA is denatured by heating the sample to 90-95^{0}C. The primers are allowed to anneal to their complementary sequence by briefly lowering the temperature to 37-60^{0}C. This is followed by heating the samples to 70-75^{0}C to extend the annealed primers with heat stable *Taq* polymerase (Chien *et al.*, 1976). The reaction mix consists of several

components, Taq DNA polymerase, deoxynucleotide triphosphates, buffer, magnesium chloride, primers and target DNA.

5.1.2 Design of 5' and 3' primers for the amplification of the α-amylase inhibitor gene.

Primers for use in the polymerase chain reaction are usually unique oligonucleotides designed from known DNA sequences. However, for an uncloned gene, such as the α-amylase inhibitor gene where no DNA sequence data is available, highly degenerate primers designed from protein sequence data may be used (Lee and Wu, 1988; Girgis *et al.*, 1988; Knoth *et al.*,1988) These redundant primers have been used before with mixed success (Lin and Brown, 1992; Wren *et al.*, 1992; Snijders *et al.*, 1992.).

Proteolytic cleavage of the α-amylase inhibitor protein had yielded a limited amount of amino acid sequence data (Ary *et al.*, 1989) which was sufficient to allow the design of PCR primers.

Back translation from the peptide to the DNA coding sequence is made complicated by the fact that most amino acids are encoded by codons degenerate at 3rd base positions. In certain cases where organisms express a bias in codon usage it is possible to design primers based on the preferential coding usage. However, at the time of this research no codon usage information was available for *Coix*. Primers were designed which would reflect the full codon redundancy in order to represent all possible peptide coding sequences.

An alternative to the complex mix of bases, is to incorporate the base inosine at positions of 3/4 base redundancies (Wilks, 1989; Aarts *et al.*, 1991). Several groups in Durham have tried inosine primers with some success (Fordham-Skelton *et al.*, 1990).

Two 17 base oligomers (primers 376 and 377) were designed from the distal C and N regions of the α-amylase inhibitor partial peptide sequence (Ary *et al.*, 1991). The sequences of the two oligonucleotide primers are shown in Figure 5.1. The position of these oligonucleotides with respect to the partial peptide sequence is illustrated in Figure 5.2. The oligonucleotides were degenerate sense and antisense to provide sense and antisense primers, such that PCR would result in the amplification of a specific DNA fragment of the α-amylase inhibitor gene. The ends of the PCR product would be defined by the 5' end of each primer. An additional oligonucleotide, primer 392 (see Figure 5.1) was synthesised to act as an internal probe to verify the correct DNA PCR products (synthesis of PCR primers is described in section 2.29).

Figure 5.1 Design of PCR oligonucleotides primers.

Three 17 base oligonucleotides were designed from the partial peptides sequence of the α-amylase inhibitor from *Coix-lachryma-jobi.* All three primers were used for attempted amplification of the *Coix* α-amylase inhibitor gene.

Primer 376

Coix amino acid sequence	NH_2	K	F	G	Y	C	G	COOH
Sense strand	5'	AAA	TTC	GGA	TAC	TGC	GG	3'
		G	T	G	T	T		
				C				
				T				
Oligonucleotide sequence	5'	AAA	TTC	GGA	TAC	TGC	GG	3'
		G	T	G	T	T		
				C				
				T				

Primer 377

Coix amino acid sequence	NH_2	Q	Y	C	Q	Q	L	COOH
Sense strand	5'	CAA	TAC	TGC	CAA	CAA	CT	3'
		G	T	T	G	G		
Oligonucleotide sequence	5'	AG	TTG	TTG	GCA	GTA	TTG	3'
			C	C	A	A	C	

Primer 392

Coix amino acid sequence	NH_2	N	Y	N	Y	G	P	COOH
Oligonucleotide sequence	5'	AAC	TAC	AAC	TAC	GGA	CC	3'
		T	T	T	T	G		
		G						
		A						

Figure 5.2 The partial peptide sequence of the *Coix lachryma-jobi* α-amylase inhibitor (after Ary *et al.* 1989).

Alignment of amino acid sequences of the tryptic and chymotryptic peptides of the *Coix* α-amylase inhibitor/endochitinase with those reported for basic endochitinases from barley (Leah *et al.* 1987), bean (Nasser *et al.* 1988), potato (Gaynor *et al.* 1988) and for basic and acidic endochitinases from tobacco. The sequence are aligned to maximise homology, resulting in the introduction of some gaps. Standard single letter abbrievation is used.
The peptide sequences choosen for the designing of PCR primers 376, 392 and 377 are marked in bold.

```
                          Residue number
          1                                                50
Bean      EQCGRQAGGALCPGGNCCSQFGWCGSTTDYC-PG-CQSQC--GGPSPAPT
Potato    QNCGSQGGGKACASGQCCSKFGWCGNTNDYCGSGNCQSQCPGGGPGPGPG
Tobacco   EQCGSQAGGARCASGLCCSKFGWCCNTNDYCGPGNCQSQCPGG-PTPPGG
Coix                      CCSKFGYCGLTDAY
                              376

          51                                              100
Barley    *SVSSIVSRAQFDRMLLHRNDGATQAKGFYTYDAFVAAAAAFPGFGRTGS
Bean      -DLSALISRSTFDQMLKHRNDGACPAKGFYTYDAFIAAAKAYPSFGNTGD
Potato    GDLGSAISNSMIDQMLKHRNLNSCQCKNFYSYNAFINAARSFPGFGTSGD
Tobacco   GDLGSIISSSMFDQMLKHRNDNACQGKGFYSYNAFINAARSFPGFGTSGD
Coix                                 NFYTGQLTS

          101
Barley    ADARK****                                       150
Bean      TATRKREIAAFLGQTSHETTGGWATAPDGPYAWGYC-FYRER-NPSTYC-
Potato    INARKREIAAFFAGTGHLTTGGWASAPDGPYAWGYC-FLRERGNPGDYC-
Tobacco   TTARKREIAAFFAQTSHETTGGWATAPDGPYAWGYCWL-REQGSPGDYCT
Coix                 FAHVTHETG                               NNAYCD

          151                                             200
Bean      SATPQFPCAPGQQYYGPGPIQISWNYNYCQCGRAIGVDLLNKPDLVATDS
Potato    PPSSQWPCAPGRKYFGPGPIQISHNYNYGPCGRAIGVDLLNNPDLVATDP
Tobacco   PS-GQWPCAPGRKYFGRGPIQISHNYNYGPCGRAIGVDLLNNPDLVATDP
Tobacco                                             ***VATDP
Coix      PSKTQKPCAAGKKYYGRGPIQISXNYNYGPCGRAIGMDGLGNPDRVAQDA
                                   392

Barley             **MTAQPPKPSSHAVIAGQWSPDGADRAAGRVPGFGVITNI
Bean      VISFKSALWFWMTAQSPKPSSHDVITSRWTPSSADVAARRLPGYGTVTNI
Potato    VISFKTALW--MTPQSPKPSCHDVIIGRWNPSSADRAANRLPGFGVITNI
Tobacco   VIS-KSALWFWMTPQSPKPSCHDVIIGRWQPSSADRAANRLPGFGVITNI
Tobacco   VISFKSALWFWMTPQSPKPSCHDVIIGRWQPSSADRAANRLPGFGVITNI
Coix      LDDYKTALXFIVNGEEAVPG             LSAANA

          251                                             300
Barley    IN****
Bean      INGGLECGRGQDSRVQDRIGFFKRYCDLLGVGYGNNLDCYSQTPFGNSLL
Potato    INGGLECGRGTDNRVQDRIGFYRRYCSILGVTPGDNLDCVNQRWFGNALL
Tobacco   INGGLECGRGTDSRVQDRIGFYRRYCSILGVSPGDNLDCGNQRSFGNGLL
Tobacco   INGGLECGRGTDSRVQDRIGFYRRYCSILGVSPGDNLDCGNQRSFGNGLL
Coix                        VSYYRQYCQQLGVDPGPNL
                                   377

          301
Bean      LSDLVTSQ*****
Potato    VDTL*****
Tobacco   VDTM*****
Tobacco   VDTM*****
```

Choices of primer sites were limited to long stretches of sequenced peptide fragments so that continuity of bases in the primer sequence was ensured. There are several points to consider when designing PCR primers (Erlich *et al.*, 1989);

1. The amino acids leucine, arginine and serine were avoided when designing the primer as they are encoded by 6 codons.

2. The primers must not contain stretches of sequence complementary to each other. In particular, primers with 3' overlaps were avoided as they can form "primer dimers".

3. They should not contain sequences which may form secondary structures particularly at their 3' ends.

4. Oligonucleotide primers are generally 17-30 bases in length.

5.2 Choice of template DNA for amplification.

Using the 2 oligonucleotide primers designed from the partial protein sequence of the α-amylase inhibitor, amplification using the PCR was attempted on 3 templates DNA's;

1. cDNA synthesised from polyA$^+$ RNA isolated from the seeds of *Coix lachryma-jobi*. (section 2.19.1)

2. Genomic DNA isolated from leaves of *Coix lachryma-jobi* (section 2.12).

3. A λgt11 cDNA library constructed using poly A$^+$ RNA isolated from the seeds of *Coix lachryma-jobi* (section 2.19)

5.3 Attempted amplification of the α-amylase-inhibitor gene from *Coix* cDNA.

In vitro translations using polyA$^+$ RNA from *Coix* seeds (middle to late stages of development) were carried out. Immunoprecipitation of these *in vitro* translation products with an antibody raised against the α-amylase inhibitor antibody had shown that the messenger RNA encoding for the α-amylase inhibitor protein was present in the RNA (personal communication M. Ary, Rothamstead Experimental Station.). Additionally, the template cDNA synthesised from this polyA$^+$ RNA was of an adequate length and integrity to contain both the primer sites. PCR with the cDNA should result in the amplification of the required product.

5.3.1 Estimation of the size of the α-amylase inhibitor PCR product.

Prediction of the size of the PCR product from the peptide map is possible as the amplification is from cDNA (no introns present). By examining the peptide map (see Figure 5.2), the primers designed are close to the C and N termini and should amplify most of the full length α-amylase inhibitor gene. The molecular weight of the native α-amylase inhibitor protein is 52,500Da, consisting of 2 identical 26,400Da subunits (Ary *et al.*, 1991). By examining the peptide map and the position of the α-amylase inhibitor primer pair, 260 amino acids lie between the 5' ends of these primers. Amplification of this sequence would correspond to 780bp. However, this estimation is dependant on the size similiarity of the *Coix* α-amylase inhibitor with the endochitinases in the peptide map. Alternatively, taking into account that the average molecular weight of an amino acid is 110Da, the 26,400Da α-amylase inhibitor subunit contains 240 amino acids. This is equivalent to 720bp DNA fragment.

5.3.2 1st strand cDNA synthesis from *Coix* seed poly A^+ RNA.

1st strand cDNA was synthesised using polyA^+ RNA from the seeds of *Coix* , mid to late stages of development (section 2.19.1).

To monitor the yield and quality of 1st strand DNA synthesis, 3μl of the 1st strand reaction mix was electrophoresed in a 0.8% agarose gel at 100v for 4 hours (section 2.10.10). A smear of cDNA molecules was visible under ultaviolet light, with the upper length of the cDNA smear being approximately 2.84kb and the lower limit .216kb (see Figure 5.3).

Therefore it was concluded that the cDNA was of adequate length for amplification.

5.3.3 Amplification from cDNA using standard conditions.

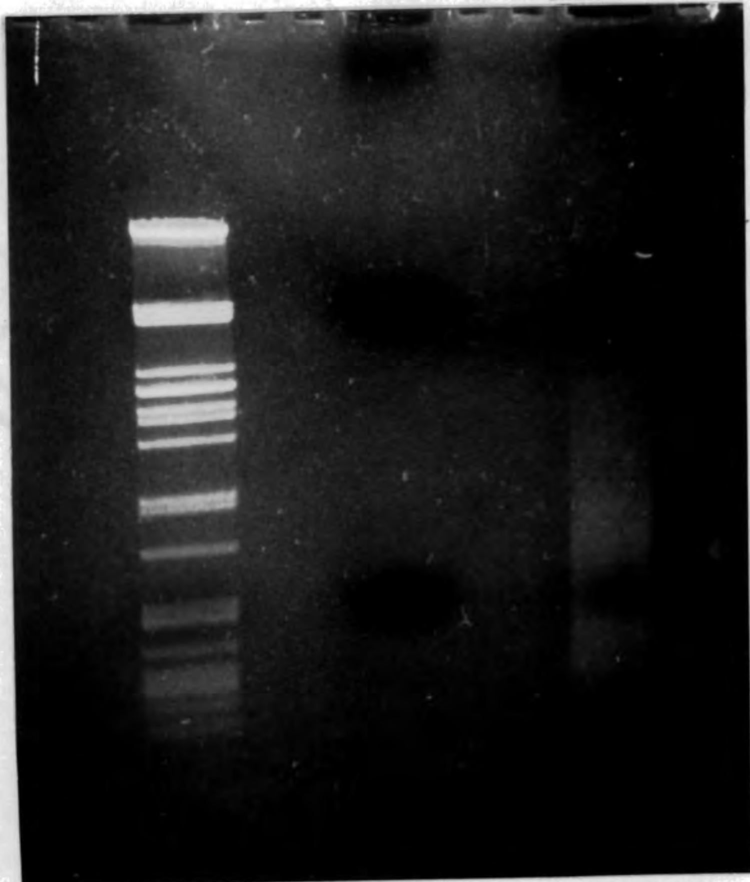
10μl of the 1st strand cDNA synthesis reaction mix was amplified (Frohman *et al.*, 1988) using 5μg of each 5' and 3' primer in a total volume of 100μl using the manufacturers buffer (section 2.25). The amplification programme is described below;

	Temperature (^{0}C)	Time (minutes)
Template denaturation	94	1.5
Primer annealing	45/40/37	2.5
Primer extension	72	3.0

Figure 5.3 Analysis of 1st strand cDNA synthesis.

Using polyA+ RNA from *Coix* seeds, 1st strand cDNA was synthesised as described in section 2.19.1. An aliquot of this was electrophoresised on a 0.8% DNA agarose gel, see section 2.10.10.

Lane 1	λPst I DNA markers
Lane 2	Unincorpoated dNTP's
Lane 3	3μl of first strand cDNA synthesis reaction.


1
2
3
kb
2.84
.216

For 30 cycles, followed by a final cycle identical to the above but with extension for 5 minutes.

A control reaction with no cDNA was also amplified to check for contaminating DNA.

Following completion of the programme 20µl aliquot were electrophoresed on a 0.8% agarose gel (2.10.10). No specific PCR products were obtained (see Figure 5.4, lanes 4 and 5).

As amplification of the template cDNA did not occur with standard conditions, several modifications to the reaction were undertaken.

5.3.4 Amplification from cDNA, varying the Mg^{2+} buffer concentration.

Several reaction buffer formulations have been published but the standard $MgCl_2$ final concentration is 1.5mM (McPherson *et al.*, 1991). For any pair of PCR primers, an optimal set of conditions can be established. In some circumstances, different concentration of Mg^{2+} may be necessary for succesful amplification. It has been reported that titrating the $MgCl_2$ concentration in the reaction buffer can affect annealing of the primer to the template thus affecting the specificity and yield of amplification. Generally, excess Mg^{2+} will result in the accumulation of non specific amplification products and insufficient Mg^{2+} will reduce the yield (McPherson *et al.*, 1991). On this basis it was decided to titrate the final $MgCl_2$ concentration in the reaction buffer from 0.5mM to 10mM. 10 X reaction buffers were made up with the following $MgCl_2$ concentrations, 5, 10, 15, 20, 25, 30, 40, 60, 80 and 100mM and amplification of cDNA using these Mg^{2+} concentrations carried out at annealing temperatures of 37^oC and 45^oC. (section 2.25).

No specific PCR products were produced but a DNA smear consisting of non specific annealing and priming products was observed following electrophoresis of the PCR products in a 0.8% agarose gel (section 2.10.10), see Figure 5.5. The DNA smearing is a result of a PCR primer annealing to the template and extending until the extended primer "drops off" the template DNA. As the extent of extension is random, various lengths of product are synthesised resulting in the smear obtained following electrophoresis of the PCR products. The extent of smearing appeared to increase to an optimum at 2.5 to 3.0mM final Mg^{2+} concentration and then decrease again as the Mg^{2+} concentration increased to 10.0mM. The extent of smearing did decrease when the annealing temperature increased to 45^oC (data not shown).

5.3.5 Amplification from cDNA with reduced primer concentrations.

Figure 5.4 Amplification by PCR of *Coix* genomic DNA and cDNA using standard reaction conditions

PCR was carried out on both templates at an annealing temperature of $40^{0}C$ with 5μg of each primer (376 and 377) using the manufacturers recomendations. PCR products were analysed by DNA agarose gel electrophoresis.

Lane 1	*Coix* genomic DNA
Lane 2	No template DNA control
Lane 3	λPst I DNA markers
Lane 4	*Coix* cDNA
Lane 5	No template DNA control

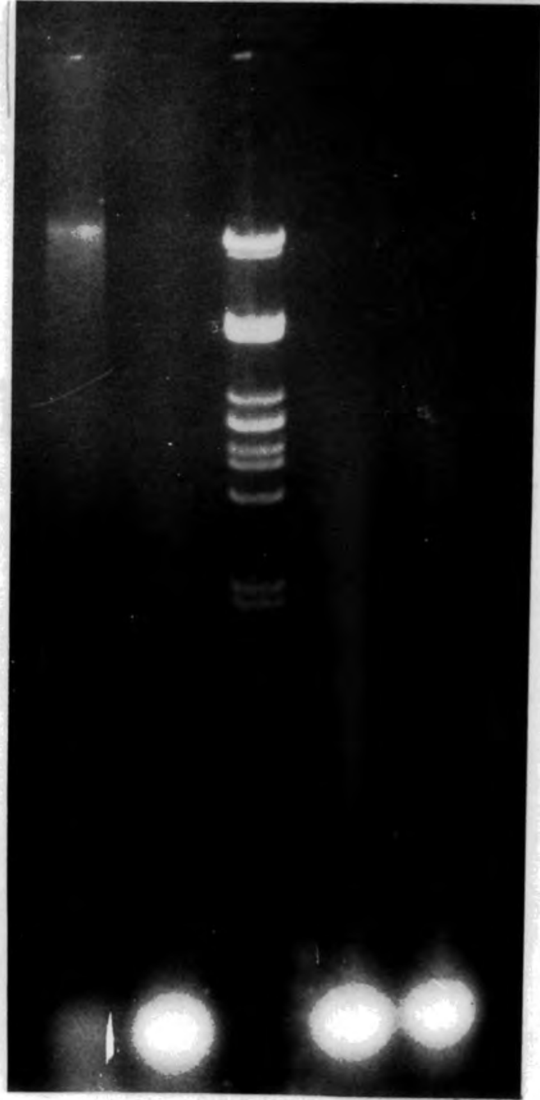
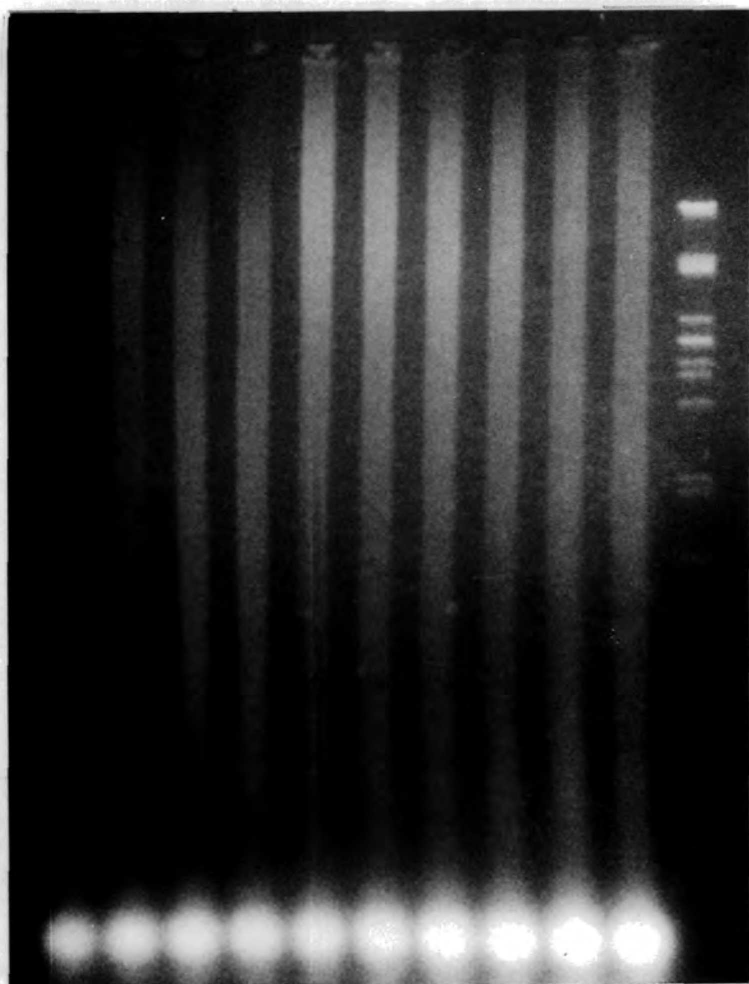

1 2 3 4 5
Template DNA
PCR primers

Figure 5.5 Analysis of PCR products following amplification of seed cDNA in varying Mg^{2+} and high primer concentrations.

Following synthesis of cDNA from seed poly A^+ RNA, amplifications were carried out using PCR primers p376 and p377 in a range of Mg^{2+} concentrations, using primer concentrations of 5μg and annealing at 37^0C.

Lane	Final Mg^{2+} concentration in reaction buffer (mM)
1	0.5
2	1.0
3	1.5
4	2.0
5	2.5
6	3.0
7	4.0
8	6.0
9	8.0
10	10.0
11	λPst I DNA markers


1 2 3 4 5 6 7 8 9 10 11
PCR primers

18μM (5μg for a 17 base oligonucleotide) of each 5' and 3' degenerate primer pool, in a 100μl reaction volume, is the standard reaction conditions which researchers in Durham University use (personal communication Dr. Robinson, Dr. Fordham-Skelton). Other researchers have had success with primer concentrations as low as 1μM (278ng for a 17mer oligonucleotide). It was decided to drop the primer concentration to 1μM final concentration and amplify over the range of $MgCl_2$ buffer concentrations used previously (see section 2.25). No specific PCR products were obtained at an annealing temperature of 37^{0}C (result not presented).

5.4 Attempted amplification of the α-amylase inhibitor gene from *Coix* genomic DNA.

5.4.1 Preparation of genomic DNA from *Coix* leaves.

Genomic DNA was isolated from the leaves of 2 week old *Coix* plants (section 2.12). The main requirements for template DNA is that the DNA should be intact over the length which is to be amplified and that inhibitors of the reaction (e.g. detergent, EDTA, traces of phenol) are not present. The genomic DNA was purified on a caesium chloride gradient and thus should not have any inhibitors present (see 2.8.2.1). Additionally, the leaf DNA was mainly in the 50kb size range as judged by pulse field electrophoresis (results not presented).

5.4.2 Amplification from genomic DNA using standard PCR conditions.

500ng of genomic DNA was amplified using standard buffer conditions, over the range of $MgCl_2$ buffers used previously (section 2.25). Using 18μM (final concentration) of each 17base oligonucleotide primer and annealing at 40^{0}C, as with the cDNA template no products were obtained (see Figure 5.4, lanes 1 and 2).

However, when the final primer concentration was decreased to 1μM (278ng) several products were amplified as described below.

5.4.3 Amplification from genomic DNA, with reduced primer concentrations.

500ng of template DNA was amplified with the α-amylase inhibitor 5' and 3' primers at a final concentration of 1μM using a range of $MgCl_2$ concentrations.

The amplification programme is described below;

Temperature (^{0}C)	Time (minutes)

92	1.5
45/50/55	2.0
72	3.0

The above was repeated for 32 cycles and then followed by a final cycle identical to the above programme but extending at 72^{0}C for 5 minutes.

20μl from each PCR reaction was electrophoresed on a 1% agarose/TAE gel system and the gel examined under ultra-violet light (section 2.10.10).

5.4.3.1 Amplification products produced at an annealing temperature of 45^{0}C.

Several products were obtained over an extensive size range (see Figure 5.6). Annealing at 45^{0}C is a relatively low annealing temperature and one would expect a certain amount of mispriming to have occured to synthesise some of these products. The amount of DNA smearing i.e. non specific priming, increases with increasing Mg^{2+} concentration reaching an optimum at 0.4 to 0.8mM final Mg^{2+} reaction buffer concentration. There is a similiar increase in the number and yield of PCR products amplified. In summary, there are 4 main PCR products amplified over the range of buffers concentrations, with sizes estimated as 1.2, 1.0, .920 and .700kb.

5.4.3.2 Amplification products produced at annealing temperatures of 50 and 55^{0}C.

In an attempt to reduce the mispriming occuring at an annealing temperature of 45^{0}C, PCR was carried out at annealing temperatures of 50 and 55^{0}C. The PCR products were analysed by gel electrophoresis as shown in Figures 5.7 and 5.8 respectively.

Template DNA was amplified as described in 2.25. Annealing at these higher temperatures resulted in a decrease in the number of different products amplified and a significant decrease in the extent of 'smearing'. This is due to priming ocurring under higher stringency conditions and so the number of misprimed products should be decreased. 4 main bands were generated by these amplifications, their occurance and yield dependant on the $MgCl_2$ concentrations, the sizes were estimated as 1.2kb, 1.0kb, 920b and 700b. It was noted that amplification of the 1.0kb PCR product was very variable.

55^{0}C is the optimum annealing temperature for the primer pair as annnealing at 60^{0}C resulted in no products (results not presented). All subsequent amplifications from genomic DNA were carried out an annealing temperture of 55^{0}C.

Figure 5.6 Analysis of PCR products following amplification of *Coix* genomic DNA at an annealing temperature of 45^{0}C.

Genomic DNA was amplified using PCR primers p376 and p377 in a range of Mg^{2+} concentrations, using final primer concentrations of 1μM and annealing at 45^{0}C.

Lane	Final Mg^{2+} concentration in reaction buffer (mM)
1	0.5
2	1.0
3	1.5
4	2.0
5	2.5
6	3.0
7	4.0
8	6.0
9	8.0
10	10.0
11	Reaction contained manufacturers buffer.
12	λPst I DNA markers

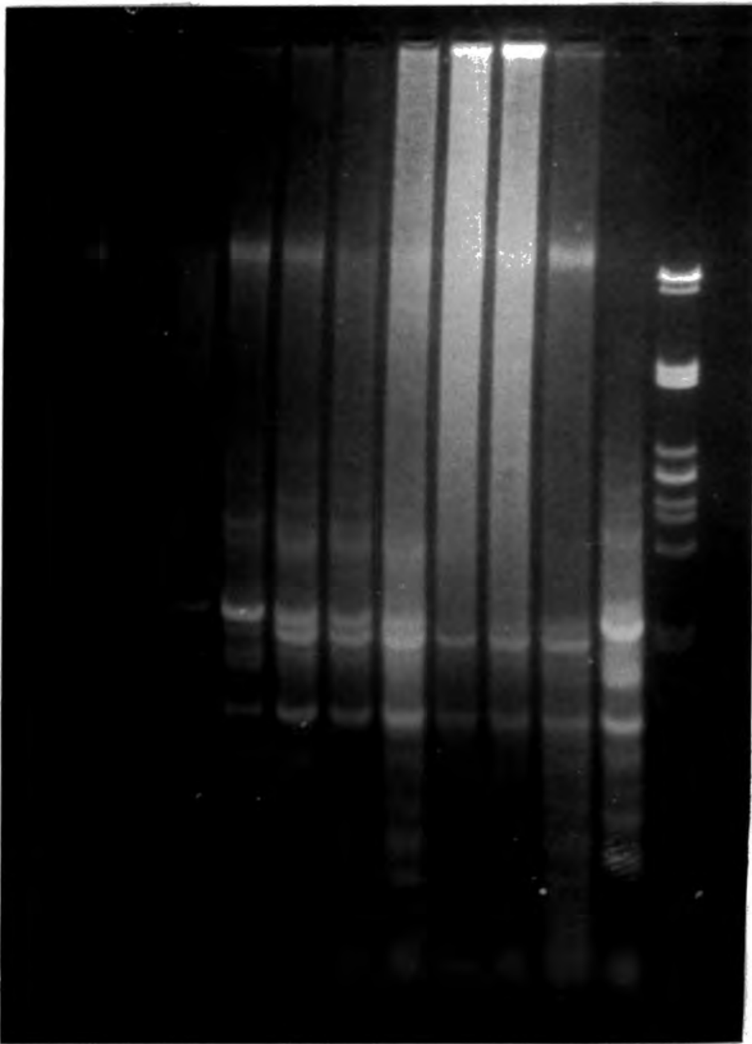
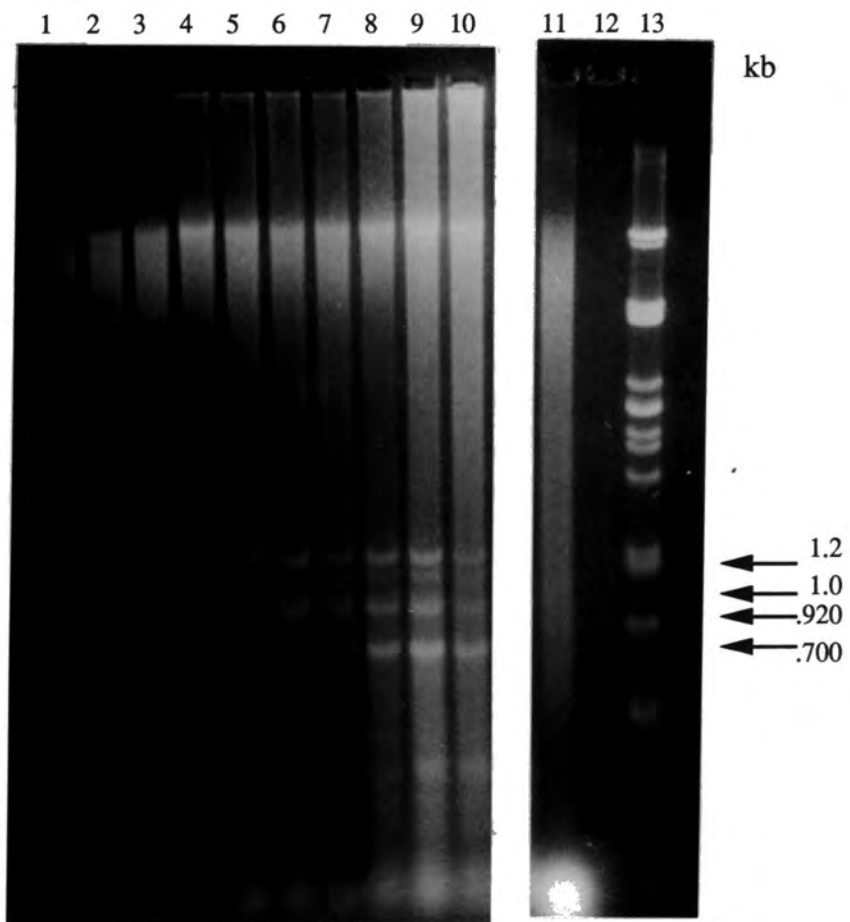

1
2
3
4
5
6
7
8
9
10
11
12
kb
Template DNA
1.2
1.0
.920
.700

Figure 5.7 Analysis of PCR products following amplification of *Coix* genomic DNA at an annealing temperature of 50^{0}C.

Genomic DNA was amplified using PCR primers p376 and p377 in a range of Mg^{2+} concentrations, using final primer concentrations of 1μM and annealing at 50^{0}C.

Lane	Final Mg^{2+} concentration in reaction buffer (mM)
1	0.5
2	1.0
3	1.5
4	2.0
5	2.5
6	3.0
7	4.0
8	6.0
9	8.0
10	10.0
11	5μg each primer, standard Mg^{2+} concentrartion.
12	No DNA control, standard Mg^{2+} concentration.
13	λPst I DNA markers

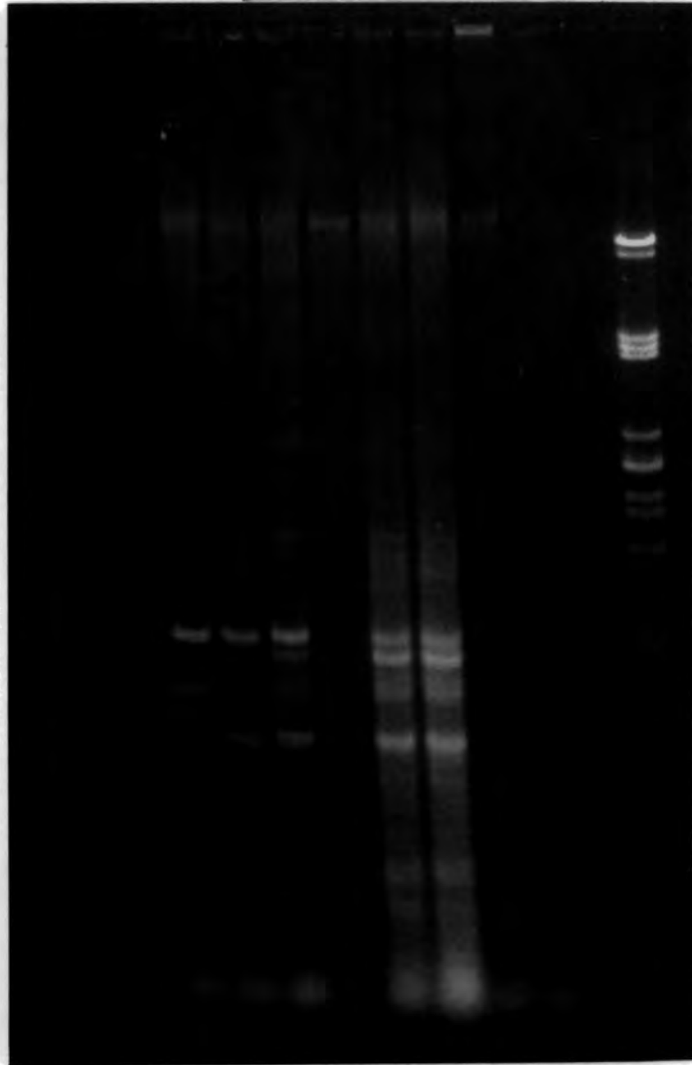


1 2 3 4 5 6 7 8 9 10
11 12 13
kb
1.2
1.0
.920
.700

Figure 5.8 Analysis of PCR products following amplification of *Coix* genomic DNA at an annealing temperature of 55^{0}C.

Genomic DNA was amplified using PCR primers p376 and p377 in a range of Mg^{2+} concentrations, using final primer concentrations of 1μM and annealing at 55^{0}C.

Lane	Final Mg^{2+} concentration in reaction buffer (mM)
1	0.5
2	1.0
3	1.5
4	2.0
5	2.5
6	3.0
7	4.0
8	6.0
9	8.0
10	10.0
11	Blank lane
12	λPst I DNA markers



1 2 3 4 5 6 7 8 9 10 11 12
kb
Template DNA
1.2
1.0
.920
.700

5.5 The effect of reducing the number of cycles in the PCR programme.

The PCR reaction amplification is not infinite. After a certain number of cycles the desired amplification fragment gradually stops accumulating exponentially and reaches a stationary phase.

For genomic DNA under standard reaction conditions, amplification using 40 cycles is recommended. However, this is dependant on the concentration of target DNA. Erlich *et al.* (1989) and Bell and de Marini (1991) reported that PCR, reactions with genomic DNA, greater than 30 cycles often resulted in reduced amounts of specific product and also produced smears on the agarose gels.

The standard programme used for the amplification of *Coix* genomic DNA consisted of 31 cycles. In an attempt to reduce the DNA smearing and increase the yields of the 4 major PCR products, it was decided to amplify the *Coix* genomic DNA reducing the number of amplification cycles to 26, 21 and 16 using the reaction conditions as described in 2.25. The final concentration of $MgCl_2$ in the reaction buffer was 0.6mM. 20µl aliquots from each reaction were electrophoresed on a 1% agarose/TAE gel as shown in Figure 5.9 (section 2.10.10). The yield and the number of the 4 main products amplified under standard conditions decreased significantly as the number of cycles in the amplification reaction were decreased. Reducing the number of cycles did not affect the smears apparent on the agarose gels.

5.6 The effect of altering the cycling parameters.

PCR is performed by incubating the samples at three temperatures corresponding to the three steps in a cycle of denaturation, annealing and extension. The time of incubation at 70-75^{0}C varies according to the length of target being amplified, 1 minute for each kilobase of sequence is recommended for preliminary PCR reactions. Initially extension of *Coix* genomic DNA was for 3 minutes but to try and reduce accumulation of non specific products, the extension time was reduced. The programmes attempted are oulined below;

	Temperature (^{0}C)	Time (min)	
Programme		1	2
Denaturation	92	1.5	1.5

Annealing	50	2	1
Extension	72	2	1

The programmes above were repeated for 30 cycles and then a final cycle was run as above, but extending for 4 and 3 minutes for programme 1 and 2 respectively. 20μl from each reaction was electrophoresed on a 1% agarose gel and the products visualised under U.V. light. Altering the cycle parameters resulted in the elimination of the products synthesised by the standard reaction (results not shown).

5.7 Reamplification of PCR product.

Several attempts at subcloning the PCR products into Bluescript SK+ had been unsuccesful and so it was decided to directly sequence the PCR product. Primers used for the preceeding PCR were to be used to initiate the sequencing reaction. However, the quantity of product generated in section 5.4.3 was insufficient for subsequent manipulations. To increase the amount of product available for sequencing, a sample of the first amplification reaction was used for reamplification (McPherson *et al.*, 1991).

3 PCR's were set up and amplified as described in section 2.25. 3, 6 and 10μl aliquots from each reaction were amplified again using identical conditions to the primary reaction. 20μl from the first and second amplifications were electrophoresed on a 1% agarose/TAE gel as illustrated in Figure 5.10. The 3 products identified in the primary amplification reactions were not specifically amplified following reamplification. Instead, a smear of non specific amplifications was produced following electrophoresis. The extent of smearing increased with the amount of primary amplification reaction used for reamplification.

5.8 Probing of PCR products with an α-amylase inhibitor oligonucleotide.

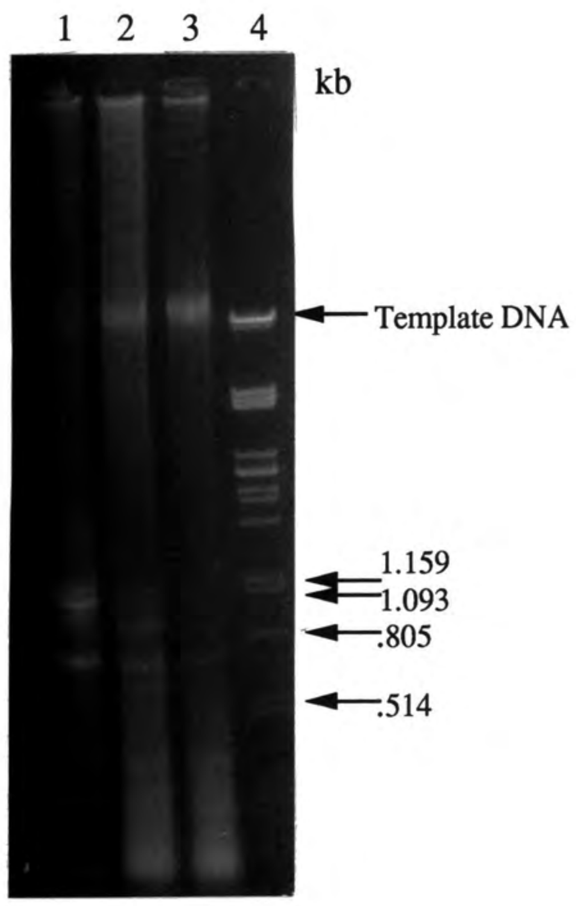
The identity of the amplification product can be verified by probing PCR products with an oligonucleotide designed from an internal region of the gene to be amplified. As the gene sequence is unknown, a degenerate 17 base oligonucleotide was designed from the peptide sequence (oligonucleotide 392). The sequence of the internal 17 oligmer is outlined in Figure 5.1 and its position in the *Coix* α-amylase inhibitor peptide sequence shown in Figure 5.2.

50ng of the the internal primer 392 (section 2.29) was labelled with ^{32}P-ATP as described in section 2.10.13.1. The labelled oligonucleotide was purified on a Sephadex G50 column and 5μl counted in a scintillation counter (section 2.10.13.3). The specific activity was calculated as 4.2×10^9 dpm μg^{-1} DNA.

Figure 5.9 The effect of reducing cycle number on the yield of specific and non specific PCR products

Coix genomic DNA was amplified at an annealing temperature of 55^0C, with primers 376 and 377 as described in section 5.5. 3 different PCR programmes were used, which varied in the number of reaction cycles. PCR products from each reaction were analysed by agarose DNA electrophoresis.

Lane 1	26 cycles
Lane 2	21 cycles
Lane 3	16 cycles
Lane 4	λPst I DNA markers


1
2
3
4
kb
Template DNA
1.159
1.093
.805
.514

Genomic DNA was amplified as described in section 2.25 and analysed by agarose gel electrophoreisis (section 2.10.10). Following electrophoresis, the DNA was transferred by capillary action to a nitrocellulose support overnight (section 2.10.15.1) and the blot probed with the labelled internal oligonucleotide 392 as described in section 2.10.15.2.

The minimum and maximum Tm of the internal oligonucleotide was calculated to allow determination of the hybridising temperatrures for probing the PCR products with the internal oligonucleotide (Sambrook *et al.*, 1989). When using a pool of oligonucleotides whose members have greatly different contents of G+C, it is impossible to estimate a consensus Tm because it is unknown which members of the pool will match the target sequence perfectly. Conditions must be used to allow the oligonucleotide with the lowest G+C to hybridise efficiently. Normally, a hybridisation temperature is choosen which is 2^0C below the calculated Tm of the most A+T rich member of the pool of oligonucleotides which in this case was 42^0C and so hybridisation was carried out at 40^0C. The blot was prehybridised for 2 hours at 40^0C, followed by hybridisation overnight at 40^0C in 25ml of hybridisation buffer containing 2.5 X 10^7 dpm. of labelled primer 392 The blot was washed in 6 X SSC, 0.1% SDS at 4^0C for 3 X 5 minutes, wrapped in cling film and then exposed for 24 hours.

The labelled oligomer was found to bind non-specifically to all of the DNA on the nitrocellulose support (results not presented), even λPst 1 DNA markers and so the filter was washed again under more stringent conditions .

The filter was washed in 6 X SSC, 0.1% SDS at room temperature for 3 X 5 minutes, wrapped in cling film and exposed for 48 hours at -80^0C. The extent of non specific binding of the probe to DNA on the filter had decreased significantly but there was no preferential binding to any of the 4 PCR products amplified (results not presented).

The filter was washed as before at 40^0C. Following exposure for 1 week at -80^0C no signal was obtained on the film (results not presented).

To try and reduce the non specific binding of the probe to DNAs on the filter the blot was reprobed at a hybridisation temperature of 45^0C and washed for 3 X 5 minutes at 4^0C. Following exposure (section 2.11), there was found to be no signal, implying that there was no hybridisation between the internal oligonucleotide and any of the DNA's on the filter (results not presented).

5.9.1 Subcloning of PCR product into Bluescript SK$^+$ cloning vector.

The 1.2kb, 1.0kb, 920b and 700kb fragments visible in Figure 5.8 were gel purified (section 2.10.12). Blunt end cloning into Hinc II digested Bluescript SK$^+$ was attempted in the following ways.

0.5μg of Bluescript SK$^+$ (section 2.3) plasmid DNA was digested with Hinc II (section 2.10.3). The gel purified PCR product (section 2.10.12) was made blunt ended with T4 DNA polymerase and ligations with SK$^+$ set up (section 2.10.7). The ligation mixes were used to transform *E.coli* DH5α (section 2.9). Transformants were selected on X-gal/Amp plates. Following restriction analysis of plasmid DNA (section 2.81) from putative transformants, it was concluded that no transformants were obtained using this technique, although positive control transformations with uncut SK$^+$ and cut Hinc II/SK$^+$ religated were satisfactory. An alternative method for cloning is described below.

5.9.2 Subcloning of the 700bp PCR product using T-vectors.

The template independent activity of Taq polymerase can be exploited to create a cloning scheme which has the efficiency of "sticky end" cloning. EcoR V digested SK$^+$ T-vector was prepared as described in section 2.6, and incubated with Taq polymerase and dTTP only. The reaction is such that the addition of a single thymine occurs in the absence of other nucleotides. The 700bp fragment was gel purified (section 2.10.12) and ligated to the EcoR V/SK$^+$ T-vector and the mix used to transform *E.coli* DH5α (section 2.9). Transformants were selected on X-gal/amp plates and white colonies were taken and restreaked. Miniprep DNA (section 2.8.1) from these colonies was double digested with Xho I and Hind II and electrophoresed on a 0.7% agarose gel (sections 2.10.1 and 2.10.10). A clone containing the 700bp PCR insert was isolated and was designated plasmid SK$^+$pcr4.

Attempts at cloning the 1.2kb, 1.0kb and 920bp fragments in the same way were unsuccessful.

5.10 Sequence analysis of the 700bp PCR product.

Template DNA was prepared for sequence analysis (section 2.27.1.1) and manually sequenced (section 2.27.1). Sequence analysis of the 700bp PCR product revealed that the primer sequence at each end of this PCR product were from the mixed pool of primers 376 as illustrated by Figure 5.11. Therefore the 700bp PCR product was formed using a single primer. The DNA sequences 3' to the priming sites were examined for homology with the α-amylase inhibitor peptide sequence but none was found.

Figure 5.11 Sequence analysis of 700bp PCR product following amplification of *Coix* genomic DNA.

Coix genomic DNA was amplified using PCR with primers 376 and 377, and annealing temperature $55^{0}C$, as described in section 5.4.3.2. The 700bp PCR product amplified was subcloned into Bluescript SK^{+} and manually sequenced (section 2.27.1). No sequence homology was found between the 700bp product and the α-amylase inhibitor. The DNA sequences of the 5' and 3' primer sites found in the 700bp product are illustrated here.

Sequence of PCR primer 376

```
5' AAA TTC GGA TAC TGC GG 3'
     G   T   G   T   T
             C
             T
```

DNA sequence of primer found in sense strand of 700bp PCR product

5' AAA TTC GGG TAT TGT GG 3'

DNA sequence of primer found in antisense strand of 700bp PCR product

5' AAG TTC GGA TAC TGT GG 3'

5.11 Amplification from *Coix* genomic DNA with single primers

It was important to determine whether the 1.2, 1kb and 920bp PCR products amplified from *Coix* genomic DNA were also single primer products. Single primer PCR reactions were carried out as described below. The PCR products amplified (section 2.25) were analysed by agarose gel electrophoresis and the DNA products visualised following ethidium bromide staining (section 2.10.10) as shown in Figure 5.12.

Primers used	PCR products generated
1. 376 Sense primer	1200/920/700bp
2. 377 Antisense primer	no products
3. 376+392 Sense and internal primers	1200/920/700bp
4. 377+392 Antisense and internal primers	no products
5. no primer control	no products

Therefore, all the PCR products generated from the original amplification reaction, which contained primers 376 and 377, were due to priming by primer 376 only. Primer 377 was not participating in amplification of these PCR products

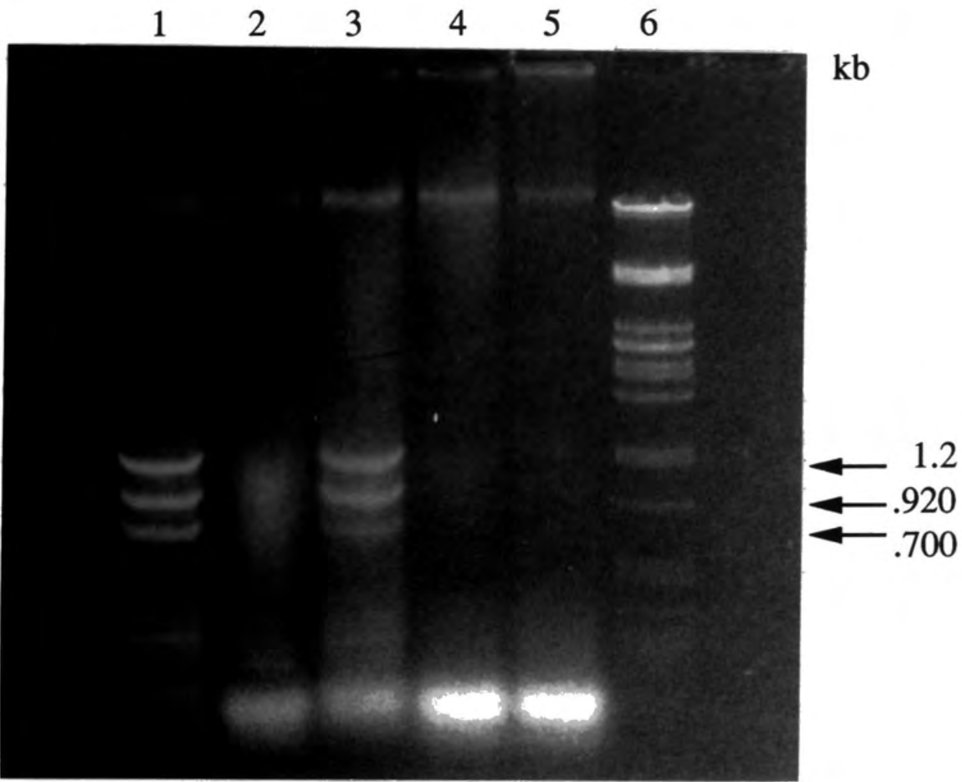
Primers 392 and 377 should constitute a primer pair because 392 is a sense primer and 377 is antisense, however no amplification products were obtained.

Theoretically there should be no amplification products resulting from PCR with primers 376 and 392 because both primers are sense primers, however products were obtained due to the mispriming of the 376 primer.

Figure 5.12 Analysis of PCR products resulting from amplification of *Coix* genomic DNA with single primers.

Amplifications of *Coix* genomic DNA were carried out as described in section 5.4.3.2, at annealing temperature of 55^{0}C. PCR products were analysed by DNA agarose gel electrophoresis.

Lane 1	*Coix* genomic DNA; primer 376
Lane 2	*Coix* genomic DNA; primer 377
Lane 3	*Coix* genomic DNA; primers 376 and 392
Lane 4	*Coix* genomic DNA; primers 377 and 392
Lane 5	*Coix* genomic DNA; primer 392
Lane 6	λPst I DNA markers


1
2
3
4
5
6
kb
1.2
.920
.700

5.12 Attempted amplification of the α-amylase inhibitor cDNA from a *Coix* λgt11 cDNA library.

5.12.1 Amplification with λgt11 primer pairs.

The rationale behind this was to to amplify the α-amylase inhibitor cDNA using primers designed from the α-amylase inhibitor sequence and from sequences flanking the cDNA insert in λgt11, as illustrated in Figure 5.13.

To check that reaction conditions were favourable for amplification of cDNA inserts from λgt11 vector, PCR was attempted using λgt11 primers 1 and 2. The titre of the phage library was calculated as 9.4 X 10^6 plaque forming units/ml. 5µl of packaged library containing 47,000 plaques was used for each amplification. This was considered to be a representative number of clones to screen for the α-amylase inhibitor gene (section 3.12.3).

Gussow and Clarkson (1989) recommend a final concentration of primers at 1µM in a total volume of 100ul using standard buffer, dNTP and Taq polymerase concentrations. Whereas Wong *et al.* (1989) recommend a final concentration of 100µM of each primer using standard PCR parameters. Phage DNA was amplified using both of these primer concentrations using the following PCR programme as described by Gussow and Wong.

	Temperature (^{0}C)	Time (mins)
Denaturation	94	1
Annealing	55	1
Extension	72	2

The programme was repeated for 30 cycles and then a final cycle carried out as above but with extension at 72^{0}C for 5 minutes. 50µl from each reaction was electrophoresed on a 1% agarose/TAE gel and the PCR products examined under ultra violet light (see Figure 5.14).

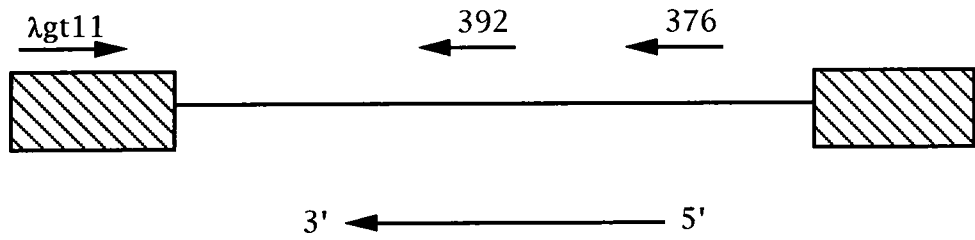
This figure shows a smear of PCR products, produced at a primer concentration of 1µM. The upper and lower size limits of the smear are defined by the lengths of the cDNAs synthesised from the poly A^+ RNA and subsequently cloned into the λgt11 vector. Amplification products up to approximately 5Kb were amplified with 1µM final concentration of primers. Using 100µM final concentration of primers, the upper limit of the size range of PCR

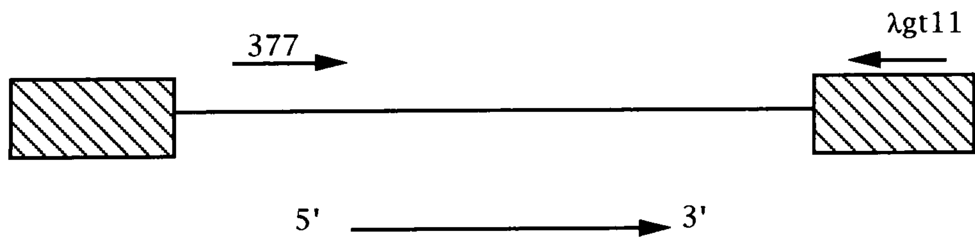
Figure 5.13 Diagram illustrating amplification of α-amylase inhibitor cDNA's from *Coix* seed λgt11 cDNA library.

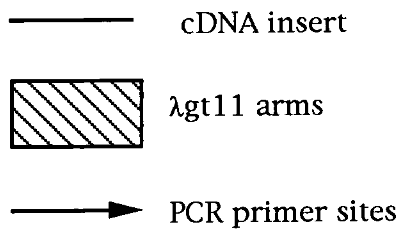
A. The strategy was to use primer pairs in which one primer was specific for the α-amylase inhibitor sequence and the other for λgt11. The α-amylase inhibitor primers used were primers 376, 377 and 392 along with λgt11 primers 1 and 2.

B. Sequence of the λgt11 primers used for PCR.

A







B

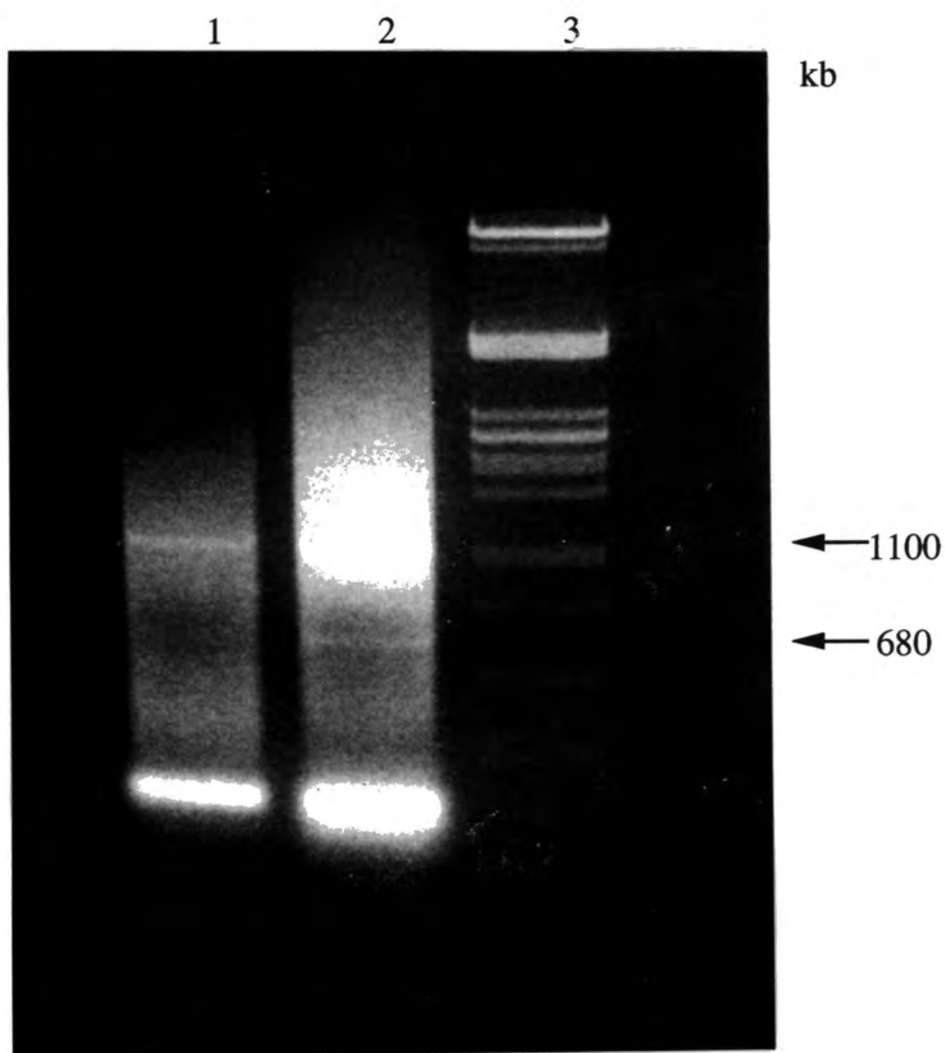
λgt11 1 GGTACGCGACCGGCGC

λgt11 2 GACTCCTGCGAGCCCG

Figure 5.14 PCR amplification of λgt11 cDNA inserts from random phage samples.

cDNA's from a *Coix* seed λgt11 cDNA libarary were amplified by PCR. Approximately 50,000pfu were used in each reaction. The primers used were designed from the λgt11 arms and amplification is described in section 5.12.1.

Lane 1	Amplification of cDNA inserts using primers λgt11 1 and 2, at a final concentration of 1μM.
Lane 2	Amplification of cDNA inserts using primers λgt11 1 and 2, at a final concentration of 100μM.
Lane 3	λPst I DNA markers


1
2
3
kb
1100
680

products amplified was approximately 14.5kb. This was suspicious as cDNAs larger than 7kb cannot be cloned and packaged in λgt11 and may be an artifact of the higher primer concentration. In addition to this, 1.10kb and 1.10 and .680kb bands were amplified in the 1μM and 100μM PCR reactions respectively. The most likely explanation is that these products are amplifications from abundant transcripts in the total RNA population.

5.12.2 Amplification with λgt11 and α-amylase inhibitor primer pairs.

As amplification of the cDNA library at 1μM primer concentrations, with the λgt11 primer pair was satisfactory, the following amplifications using the α-amylase inhibitor sequence primers 376, 377 and 392 were set up using 1μM final primer concentration (section 5.12.1). PCR products amplified were analysed by agarose gel electrophoresis (see Figure 5.15). The PCR reactions and the products generated are described below;

Primers used for PCR	Products amplified
1. primer 376 + primer 377.	no products
2. λgt11 primer 1 + primer 377.	1090, 720 and 490bp
3. λgt11 primer 2 + primer 377.	no products
4. λgt11 primer 1 + primer 376.	no products
5. λgt11 primer 2 + primer 376.	290bp
6. λgt11 primer 1 + primer 392.	1090, 720 and 490bp
7. λgt11 primer 2 + primer 392	no products
8. λ Pst I DNA markers	

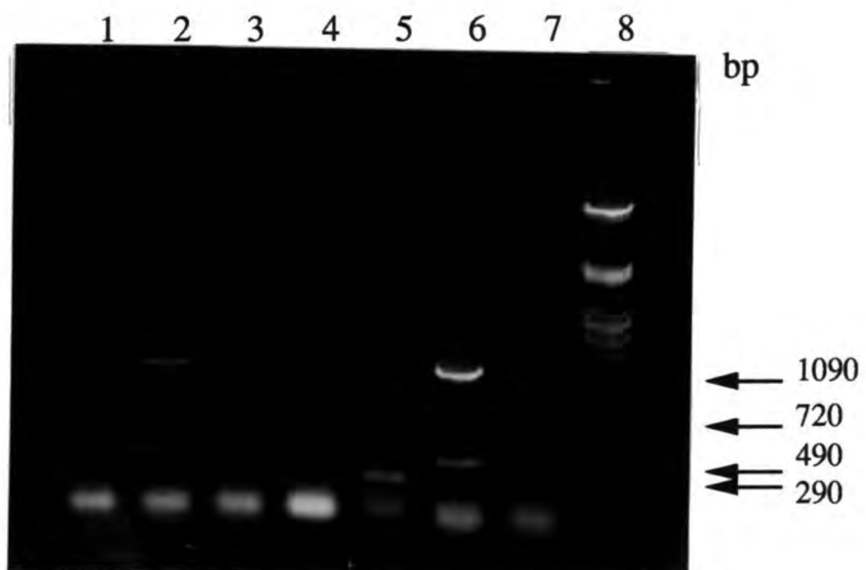
Possitive and negative controls were set up as described below;

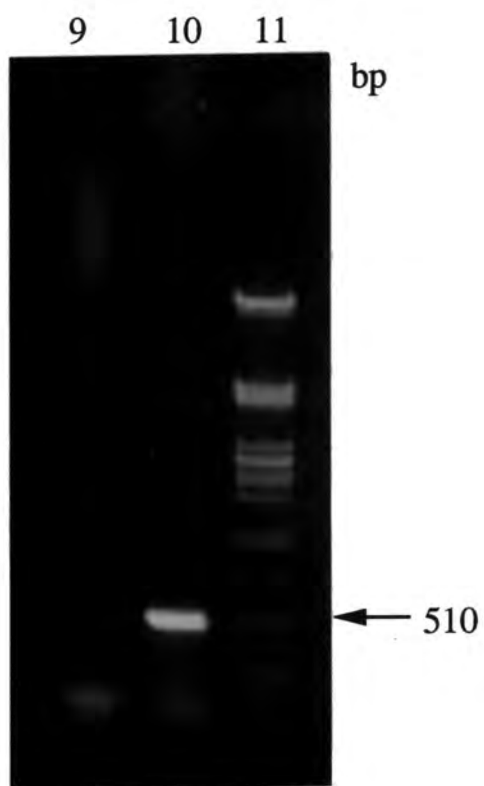
9. λgt11 primers 1 + 2 with no template DNA.	no products
10. λgt11 primers 1 + 2 with pAT_2 characterised cDNA clone	510bp
11. λ Pst I DNA markers	

Figure 5.15 PCR amplification of cDNA's from a *Coix* seed cDNA λgt11 library with λgt11 and α-amylase inhibitor PCR primers.

Random cDNA's were used as template for the attempted amplification of the α-amylase inhibitor cDNA. The primers pairs used were combinations of a λgt11 primer with an α-amylase inhibitor primer (376, 377 or 392) and are described below.

Lane 1	PCR primers 376 and 377.
Lane 2	λgt11 1 and 377.
Lane 3	λgt11 2 and 377.
Lane 4	λgt11 1 and 376.
Lane 5	λgt11 2 and 376.
Lane 6	λgt11 1 and 392.
Lane 7	λgt11 2 and 392
Lane 8	λPst1 DNA markers
Lane 9	λgt11 1 and 2, no template DNA
Lane 10	λgt11 1 and 2, characterised cDNA clone containing pAT_2, 510bp insert.
Lane 11	λPst1 DNA markers.


1
2
3
4
5
6
7
8
bp
1090
720
490
290


9
10
11
bp
510

The control amplifications were satisfactory. There was no template contamination in the PCR reaction constituents and the reaction conditions were appropriate to amplify up a 510bp product from a cDNA insert which had been previously characterised as containing an insert of that size (personal communication Dr. A. Tommie).

No products were obtained following amplification of λgt11 cDNA clones with both primers 376 and 377. Annealing of both primers to the template DNA is a prerequisite for amplification, it is possible that both primer annealing sites were not present on the same cDNA insert. The size of the α-amylase inhibitor PCR product was predicted to be 720bp (see section 5.3.1). The size range of the cDNA library was analysed in 5.12.1 and a maximum insert size of approximately 5kb generated. Therefore, the occurance of both priming sites in the same cDNA insert is probable.

5.12.3 Amplification from λgt11 cDNA library with single primers.

In addition to the above reactions, PCR amplifications with only 1 primer per reaction were carried out. PCR reactions containing the following primers were amplified using 1mM the conditions outlined in section 5.12.2, and the products analysed by agarose gel electrophoresis (see Figure 5.16).

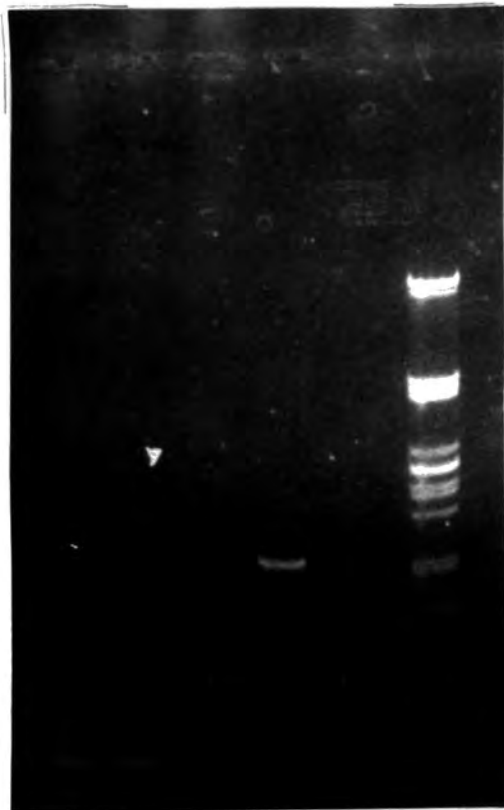
Products used for PCR	Products generated
1. p376	no products
2. p377	no products
3. p392	no products
4. λgt11 1	1090, 720, 490
5. λgt11 2	290bp

PCR products were obtained with single primers λgt11 1 and λgt11 2, suggesting that the PCR products obtained in reactions 2, 5 and 6 were products of the λgt 11 primers solely and primers 376, 377 and 392 were not required for amplification of these products.

Figure 5.16 PCR amplification of cDNA's from a *Coix* seed cDNA λgt11 library with single PCR primers.

Random cDNA's were used as template for amplification using single λgt11 or α-amylase inhibitor primers.

Lane 1	primer 376
Lane 2	primer 377
Lane 3	primer 392
Lane 4	λgt11 primer 1
Lane 5	λgt11 primer 2
Lane 6	λPst 1 DNA markers



1 2 3 4 5 6
bp
1090
720
490
290

5.13 DISCUSSION.

5.13.1 The PCR primers.

The PCR primers are probably the single most important factor in determining the success of any PCR experiment. The most apparent reason for the failure of PCR to amplify the α-amylase inhibitor gene lies with the PCR primers used for amplification. The approach to the selection of efficient and specific primers remains somewhat empirical. Although guidelines are followed there is no set of rules which will ensure the synthesis of an efficient primer pair. This is especially the case for degenerate primers. Primers used in the polymerase chain reaction are usually unique oligonucleotides designed from a known DNA sequence, and much success has been reported with these primers. Although there are many reports in the literature of succesful experiments, a greater number of problems have been encountered with the use of degenerate primers. Occasionally primers will be synthesised which completely fail to amplify up their intended target sequence. The primers used for amplification were designed from highly conserved regions of the α-amylase inhibitor peptide sequence. Assuming that the fault may lie with the PCR primers, 3 new 5' and 3' primers could have been designed from a different area of the α-amyalse inhibitor peptide sequence and amplification attempted again.

The primer pair (376 and 377) designed for amplification of the α-amylase inhibitor gene were both degenerate primers designed from the *Coix* α–amylase inhibitor partial peptide sequnce. Both primers were 17 bases long each with 64 fold redundancy, which effectively means that there are 64 different sequences with which each primer pool can anneal to. Thus succesful amplification of the correct PCR products relies on generating stringent conditions in which the primers only anneal with the desired target sequence and mismatch annealing is not feasible. McPherson *et al.* (1991) have succesfully used PCR primers ranging in potential redundancy from 64- to 73 728-fold to amplify genomic DNA from species of bacteria, fungi, plants and animals.

An alternative approach to designing degenerate primers is to substitute the universal base inosine at each redundant base (Knoth *et al.*, 1988). At present the decision of whether to use mixed pool primers rather than inosine primers is very much personal preference. However it would be interesting to attempt amplification using these primers incorporating inosine and compare the PCR products generated .

Due to the redundancy of the primers designed it was not possible to use the relevant computer software to determine whether either of these primers had any significant degree of

secondary structure. If this does occur, particularly at the 3' end, annealing can be prevented. Some PCR protocols include 10% dimethyl sulfoxide (DMSO) to reduce the secondary structure of the DNA template (Windship *et ai.*, 1989). However, several workers have reported that DMSO (at 5% v/v) can be inhibitory to Taq polymerase (as much as 50%) and decrease product yield. For these reasons it was not used.

At present there are several compounds on the market which are reported to improve the specificity of the PCR reaction by destabilising mismatched duplexes. These include the product Perfect match (stratagene) and formamide (Sarkar *et al.*, 1990).

Secondary structure of the PCR template can sometimes be inhibitory against the PCR reaction. However, the initial denaturation step in the PCR cycle of 94^0C for 1.5 minutes should destroy any cDNA/hybrids and secondary structures which could have inhibited the initial priming step (Fenton-Williams, 1987).

Saikai (1989) reported that reducing the primer concentration serves to limit mispriming and so increase specificity. Decreasing the primer concentration in the reaction mix to 1μM resulted in the generation of several PCR products. No PCR products were amplified at the higher primer concentrations. One reason for this is that DNA along with free dNTP's and some proteins appear to bind the Mg^{2+} which is present in the reaction buffer (Erlich *et al.*, 1989). The concentration of $MgCl_2$ can profoundly affect the specificity and yield of an amplification reaction. At the higher primer concenrations in which the initial amplification was attempted it is likely that the concentration of free Mg^{2+} was significantly depleted and did not meet the Mg^{2+} requirements for amplification by PCR to occur.

5.13.2 Mg^{2+} concentration in the reaction buffer.

The standard concentration of Mg^{2+} present in the reaction mix is optimal at 150μM (with 200μM of each dNTP), but in some circumstances amplifications with several titrations of Mg^{2+} may prove necessary depending on the primer pair. It has been reported that excess Mg^{2+} will result in the accumulation of non specific amplification products and insufficient Mg^{2+} will reduce the yield (Saikai, 1989). It was clearly apparent that titering the Mg^{2+} concentration in the reaction buffer had a significant effect on the nature of PCR products generated.

5.13.3 Annealing temperature.

The annealing temperature of primer to the template during the polymerase chain reaction is one of the factors which determines the stringency of the amplification. The temperature at

which annealing occurs is dependant on the length and GC content of the primer pair. As the primers used in this reaction are degenerate estimation of annealing temperatures using the standard formulas will only be an approximate. McPherson *et al.,* (1991) recommend that an annealing temperature of 55^{0}C is a good starting point for a 20 base oligonucleotide with a GC content of approximately 50%.

When designing the primers, the melting temperatures (Tm), and as such the G+C content of the primers were taken into account. The minimum and maximum Tm for each primer was calculated using the following equation (Sambrook *et al.,* 1989);

Tm= 4(G+C) + 2(T+A)

This equation is normally used when oligonucleotides are present in high salt concentrations (0.9M NaCl) and does not take into account the conditions in which most PCR takes place, however it is a good estimate. The minimum and maximum Tm for primer 376 were 44^{0}C and 65^{0}C, and for primer 377, 42^{0}C and 56^{0}C respectively both very similiar. This is extremely relevant when considering the annealing temperatures at which the PCR amplification should be carried out at. The annealing temperature along with the time period controls the annealing of the primer to the template reaction. Too high a temperature causes the primers to dissociate and there is no amplification, too low a temperature allows the primers to bind non specifically and cause amplification of non specific products. Analyses of the PCR products following amplification of *Coix* genomic DNA using primers 376 and 377 had concluded that the 4 main PCR products were single primer products. It is possible that whilst one primer anneals at a high temperature, the opposite primer may not because of of a lower melting temperature. Although this may have been the case, it is unlikely due to the similiar Tm of primers 376 and 377. Additionally these four single primer products were all apparant at an annealing temperature of 40^{0}C, which is relatively non-stringent in relation to their Tm's, and should have allowed the annealing of both primers.

5.13.4 PCR amplification with cDNA

PCR amplification of cDNA using primers 376 and 377 was unsuccesful throughout a range of reaction conditions, including high and low primer concentrations, a range of Mg^{2+} concentrations and at low annealing temperatures.

The length or integrity of the cDNA does not appear to be the reason for the failure of the PCR to amplify the α-amylase inhibitor sequence (section 5.3). In retrospect, probing of a Northern blot of *Coix* RNA or the 1st strand cDNA synthesis reaction with both of the

primers could have been carried out. This would of verified if both of the priming sites were actually present in the template cDNA.

5.13.5 PCR amplification with λgt11 cDNA library.

Using primers designed from the α-amylase inhibitor peptide sequence along with primers to sequences flanking the cDNA inserts in λgt11, amplification of the α-amylase inhibitor cDNA was attempted.

Due to the non-directional cloning of the cDNAs into λgt11 it was unknown which λgt11 primer (1 or 2) would prime with the primers internal to the inserts. However, due to the fact that both primer 376 and 392 prime in the same direction on the same template strand, it was expected that both of these primers would produce PCR products with the same λgt11 primer, and that primer 377 would prime with the other λgt11 primer. Results from amplifications carried out in section 5.12.2 suggest that this was not the case. Primers 377 and 392 primed with λgt11 1 and λgt11 2 with 376. In addition to this if, complementary sites to the primers 376 and 392 were present in the same cDNA insert then the product obtained with primer 392 should be smaller than the product obtained from 376.

A further consideration was that the cDNA was not directionally cloned into the λgt11 vector and so the priming direction of the λgt11 primers with respect to the 5' and 3' α-amylase-inhibitor primers internal to the cDNA insert was unknown. Therefore, all possible primer pair combinations of λgt11 primers with primers 376 and 377 were used for amplification of the cDNA library .

PCR products were obtained following amplification of the cDNA library with the λgt11 and α-amylase inhibitor primers section 5.12.2. Further amplifications described in section 5.12.3 implied that these PCR products were single primer products derived solely from the λgt11 primers.

However, there is an anomaly in the results of the amplifications from the λgt11 cDNA library. When amplifying λgt11 primer 2 with α-amylase inhibitor primers 377, 392 and λgt11 primer 1 with primer 376, no PCR products were obtained. This contradicts the theory that the products are single primer products. One explanation is that this may be an artifact of the PCR reaction.

Sequencing these PCR products would have determined the sequence of the PCR primers which determined amplification. Without this information it is futile to speculate on the discrepancy between these results.

5.13.6 PCR amplification with genomic DNA.

Single primer products were derived from amplification with primer pool 376. The fact that several single primer products were obtained suggests that these inverted repeats are found several times in the template genomic DNA. This target may be more abundant in the target DNA than the other primer target sequence (377), and so would be preferentially amplified, rapidly competing out any products derived from the primer pair 376 and 377.

It would have been interesting to completely sequence the 1,200, 1,000. 920 and 700bp PCR products to determine what, if anything they encoded for. The 700bp PCR product was used to probe *Coix* seed and leaf RNA (results not presented). However, preliminary results suggest that there was no hybridisation between the probe and RNA. This implies that the amplification product was not expressed in *Coix* DNA. Alternatively it may not have been expressed at the developmental stage at which the RNA was isolated from. Extensive Northern blots with seed RNA from several developmental stages could have to be carried out to clarify this.

The extent of smearing visualised following agarose gel electrophoreis of the PCR products is thought to be due to several properties of the PCR reaction. Bell and de Marini (1991) postulated that after approximately 30 cycles, most of the PCR primers have been converted into PCR product, and that at this time reaction conditions favour the annealing of the 3'OH ends of the PCR product to genomic template or to itself. The 3'OH ends of the PCR product are then extended to higher molecular weight DNA and are randomly terminated during the additional cycles. These random length products are the likely components of the smear observed on the agarose gel. Upon further cycling, the intensity of the smear increased as the specific PCR product decreased. Unfortunately, reducing the number of cycle in the PCR programme did not reduce the smearing or increase the PCR products yields.

Alternatively the smearing may be generated by primers annealing to and extending along the DNA template until it eventually "drops off" the template. These observations suggest that both primers may never have annealed to the template DNA and that the fault may lie with primer 377. Without further experiments to determine the integrity of the primer, little else may be concluded.

5.13.7 Subcloning PCR products

Despite numerous attempts to subclone the PCR products amplified from genomic DNA only the 700bp product was succesfully subcloned. Reported attempts to clone PCR products as blunt ended fragments have been very inefficient, this is thought to be due to the template-

independent terminal transferase activity of Taq polymerase, which results in the addition of a single nucleotide at the 3' end of the fragment (Clark, 1988; Mole *et al.*, 1989). This nucleotide is virtually always an adenosine, due to the strong preference of the polymerase for dATP (Clark, 1988). Hemsley *et al.* (1989) recommends that cloning the products as blunt-ended fragments requires enzymatic processing to remove the 3' overhang using an enzyme with 3' to 5' exonuclease activity.

Alternatively, restriction endonuclease sites are often incorporated into the oligonucleotide primers used for amplification. Thus cleavage of the resulting PCR product will generate sticky ends for more efficient ligation into cut vector (Scharf *et al.*, 1986). However, it was decided not to design restriction sites onto the ends of the primers to avoid any mispriming that these may have encouraged. Although there is no evidence that the addition of restriction sites at the 5' end of PCR primers effects their specificity, and a number of workers have had success using this system.

5.13.7 Further experiments.

Despite lengthy optimization procedures with the PCR reaction, amplifcation of the α-amylase inhibitor coding sequence was not achieved. The approach to isolating the α-amylase inhibitor gene utilised the standard PCR protocol of amplification with 3' and 5' primers. Recently, several other PCR protocols have been designed which could be used to isolate the α-amylase inhibitor gene.

For example, 3' and 5' RACE (rapid amplification of cDNA ends) could have been attempted (Frohman *et al.*, 1988). This allows amplification of the 5' and 3' ends of a target cDNA. Assuming that only one of the α-amylase inhibitor specific primers, 376 was annealing to the template, 3' RACE could have been attempted. This would have amplified a 3' fragment of the cDNA, which could have been used to screen the λgt11 library to isolate the full length clone.

Alternatively, there was sufficient α-amylase inhibitor peptide sequence to allow the design of nested primers. In nested PCR a reaction is performed with the outer primer pair (McPherson *et al.*, 1991; Williamson and Rybicki, 1991). An aliquot of this reaction is then reamplified with the inner pair of primers. This approach significantly increases the sensitivity of the PCR, since two pairs of primers are required to amplify the target sequence for a final product to be generated.

Another approach using PCR has recently been devised named "Touchdown PCR" (Don *et al.*, 1991). This was designed with the aim of circumventing spurious priming during gene

amplification. 'Touchdown' exploits the exponential nature of the the PCR reaction, and begins at or above, rather than below the expected annealing temperature. The annealing temperature of the reaction is decreased 1^0C every second cycle from 65^0C to a touchdown temperature at 55^0C, at which temperature 10 cycles are carried out. Any difference in the Tm between the correct and incorrect annealings will give an advantage of 2 fold per cycle, or 4 fold per ^{0}C to the correct product, all else being equal. Therefore, a 5^0C difference would give a 4^5 or 1024 fold advantage.

The authors suggest that this approach should be applicable to a wide range of situations, conveniently bypassing spurious amplifications without lengthy optimization procedures.

SUMMARY

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By exploiting the inherent resistance shown by some plants against insect herbivores and microbial pathogens, the transfer of resistance genes into agronomically important crop plants is a major goal of crop improvement. The source of these genes is the extensive plant defence mechanisms which protect plants against pests and pathogens. These include physical barriers, secondary metabolites and antimetabolic proteins.

Some of these plant defence proteins are specifically induced following invasion of the plant by its pathogens. These pathogenesis related proteins, include fungal cell wall degrading enzymes such as hydrolases, β-glucanases, lectins and chitinases (Shapira *et al.*, 1989).

Additionally, plants contain a range of proteins which act as natural inhibitors for a variety of enzymes such as trypsin, chymotrypsin, subtilisin, papain, bromelain, α-amylases and transferases. These inhibitors are found in several plant tissues including the fruits, tubers and seeds. Inhibitors of endogenous enzymes are thought to be involved in general metabolism by regulating the activity of their target proteins. Whereas, other proteins specifically inhibit exogenous enzymes, and it has been suggested that these are involved in plant defence. They are thought to be involved in the protection of the plants against tissue damage, and invasion by predators (Garcia-Olmedo, 1987). Interest in the plant α-amylase inhibitor protein family has recently increased bearing in mind their potential for use in crop improvement by genetic engineering.

An example of plant proteinaceous inhibitors conferring resistance against pathogens is illustrated by the cow pea trypsin inhibitor, a proteinase inhibitor present in the cowpea seed (*Vigna unguiculata*) a grain legume. As a result of feeding trials, Gatehouse *et al.* (1990) found the purified trypsin inhibitor to be an effective insecticide against a wide range of economically important field and storage insect pests. Some of which cause substantial financial losses to crop growers. Boulter *et al.* (1990) transformed tobacco (*Nicotiana tabacum*) with the cow pea trypsin inhibitor gene (Hilder *et al.*, 1987). Insect bioassays on the plants showed a negative effect on the growth and mortality rates of the tobacco budworm.

Ary *et al.* (1989) purified an α-amylase inhibitor from the seeds of *Coix lachryma-jobi*. The inhibitor consisted of two major isomers, each was found to be a dimer of nearly identical 26,000Da molecular weight. The major isomer was assayed for inhibitory activity against a-amylases from *Bacillus subtilis*, porcine pancreas, human saliva, *Aspergillus oryzae* and

barley malt and found to be non inhibitory. The major isomer did inhibit α-amylase from *Locusta migratora migratoriodes* (African locust). Additionally, the partial amino acid sequence of the major isoform revealed a high homology with partial and complete amino acid sequences of endochitinases from a number of sources including barley seeds and the leaves of tobacco, potato and bean. Endochitinase activity was demonstrated by following the release of radioactivity from tritiated labelled chitin. This novel combination of enzymatic and inhibitor functions suggests a possible role of this protein in protecting the plant against deleterious insect and fungal invasion. It would be interesting to carry out further feeding trials to assess the effect of this protein on other insect and fungal plant pathogens.

The aim of this research was to isolate the α-amylase inhibitor/endochitinase cDNA and genomic clones. Three strategies were adopted for isolation of this gene.

A λgt11 cDNA expression library was constructed from *Coix* seed polyA$^+$ RNA with the aim of isolating the gene of interest by immunoscreening this library. Two antibodies were available for screening, one raised against the *Coix* α-amylase inhibitor/endochitinase protein and the other against an endochitinase from wheat germ (gift of M. Ary). Both had been previously characterised by M. Ary as cross reacting against the α-amylase inhibitor/endochitinase protein from *Coix* seeds.

Immunoscreening with the *Coix* α-amylase inhibitor antibodies did not result in the isolation of any immunopositive clones. However, three immunopositive cDNA clones were isolated following immunoscreening with the endochitinase antibodies, namely pDF2.2, pDF5.1 and pDF3.17. Sequencing of all three clones was attempted. pDF5.1 and pDF3.17 cDNAs have been sequenced and pDF3.17 found to be a seed storage protein called α-coixin, which are very similar to the zein storage proteins in maize. The nucleotide sequence and deduced amino acid sequence of pDF5.1 cDNA were analysed by comparison with the EMBL databases. No significant sequence similarity was found in either case.

Due to the failure of previous immunoscreening strategies to isolate the gene of interest, it was decided to raise polyclonal antibodies against a glutathione-S-transferase-α-amylase inhibitor fusion peptide. The antigen was isolated following expression of an α-amylase inhibitor peptide encoding insert, in the bacterial expression vector pGEX3X. Polyclonal antibodies were successfully elicited in mice against glutathione-S-transferase. However Western blots following probing of *Coix* seed proteins with these antibodies suggested that antibodies had not been elicited against the *Coix* α-amylase inhibitor peptide.

A relatively recent approach for gene isolation, is amplification of the gene of interest using the polymerase chain reaction. Amplification of the α-amylase inhibitor/endochitinase cDNA and genomic sequences was attempted from cDNA, genomic DNA and a cDNA library. PCR primers used for amplification were designed from the C and N termini of the α-amylase inhibitor/endochitinase partial peptide sequence. PCR products were amplified from genomic DNA and the cDNA library. Further characterisation of these products revealed that none were α-amylase inhibitor products. Despite lengthy optimisation procedures, to increase the stringency of amplification, the α-amylase inhibitor gene was not amplified.

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