Digestive enzymes of vine weevil (Otiorhynchus sulcatus) as potential targets for insect control strategies.

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Abstract

Over the previous quarter century the vine weevil (*Otiorhynchus sulcatus*) has become a pest of horticultural and agricultural plants. The vine weevil is a polyphagous coleopteran insect and is able to attack over one hundred different plant species. Its spread has been limited by its lack of flight but modern world trade in live container grown plants has spread the insect to new habitats. Damage to plants caused by vine weevil is two fold, with the larvae destroying root balls while the adults attack the leaves. The larval stage, in particular is difficult to treat with conventional insecticides unless environmentally undesirable soil treatments are used. The current lack of defence against the vine weevil has opened the door for methods of crop protection through the generation of genetically modified plants.

The design of an efficient GM approach to control the vine weevil requires a sound knowledge of the insect's digestive enzymes, which may be used as potential targets for insecticidal proteins. This approach was achieved for the vine weevil through analysis of active digestive proteases in the insects gut and the identification of suitable proteinase inhibitors which would reduce the overall level of protein hydrolysis. Using this method it was discovered that the vine weevil contained both serine and cysteine proteases in addition to a range of other digestive hydrolases.

This biochemical data was supported by a molecular approach to isolate cDNA clones associated with the insect's digestive tract. Using a gut specific cDNA library clones encoding a cathepsin B protease, two trypsin proteases, a pectinesterase, a lipase and a cellulase were isolated and characterised.

The cellulase isolated from vine weevil has been shown to originate from the insect genome as shown through Southern Blot analysis and sequencing across several intronic regions. Evidence presented herein shows that the vine weevil gut extract hydrolyses both cellulose and cellobiose. Similar results were observed with recombinant protein expressed in the eukaryotic yeast *P.pastoris*. Furthermore data presented here shows that the vine weevil has the full complement of enzymes needed for the complete digestion of crystalline cellulose, which was until recently believed to be the sole domain of several species of bacteria and yeast.

In addition a cDNA clone encoded a vine weevil endogenous chitinase was isolated from the cDNA library. This chitinase cDNA and one encoding the proteinase inhibitor Oryzacystatin-I were used to generate transgenic tobacco plants which have been shown to express the transgene. These transgenic plants are the first step in developing a strategy for plant protection against vine weevil based on genetic modification.

Declaration

No part of this thesis has been previously submitted for a degree in this or any other University. I declare that, unless otherwise stated, the work presented herein is entirely my own.

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Digestive Enzymes of Vine Weevil (*Otiorhynchus sulcatus*) as Potential Targets for Insect Control Strategies.

A thesis submitted by Martin Gethin Edwards, B.Sc., M.Sc., in accordance with the requirements of the University of Durham for the degree of Doctor of Philosophy.

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Abbreviations

bis-acrylamide	-	bis (*N,N'*-methylene-bis-acrylamide
bp	-	base pair (nucleic acid)
BSA	-	bovine serum albumin
cDNA	-	complementary DNA
Ci	-	Curie
cpm	-	counts per minute
DEPC	-	diethylpyrocarbamate
DMSO	-	dimethylsulfoxide
DMF	-	dimethylformamide
DNA	-	deoxyribonucleic acid
DTT	-	dithiothreitol
ECL	-	enhanced chemiluminescence
EDTA	-	ethylene diamine tetraacetic acid
EtBr	-	ethidium bromide
HGT (agarose)	-	high gel temperature agarose
IPM	-	integrated pest management
IPTG	-	isopropyl-ß-D-thiogalactoside
kbp	-	kilo base pair
kDa	-	kilo Dalton
M_r	-	molecular weight
mRNA	-	messenger RNA
OC-I	-	oryzacystatin I
OD	-	optical density
O/N	-	over-night
ORF	-	open reading frame
PAGE	-	polyacrylamide gel electrophoresis
PBS	-	phosphate-buffered saline
PCR	-	polymerase chain reaction
pfu	-	plaque forming units
rcf	-	relative centripetal force
RNA	-	ribonucleic acid
rpm	-	revolutions per minute
RT	-	reverse transcription
rt	-	room temperature
SDS	-	sodium dodecyl sulphate
SDS-PAGE	-	sodium dodecyl sulphate polyacrylamide gel electrophoresis

SSC	-	saline sodium citrate
TAE	-	DNA gel electrophoresis buffer
Temed	-	*N,N,N',N'*-tetramethylethylenediamine
UTR	-	untranslated region, either 5' or 3'
v/v	-	volume/volume
w/v	-	weight/volume
X-Gal	-	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

1 Introduction

Members of the genus *Otiorhynchus* are polyphagous arthropod pests that can be found in many parts of the world. The *otiorhynchids* are believed to have originated from Europe and it has been estimated that the family contains over 1000 species (Wilcox *et al*, 1934), with fossil evidence dating the origin of the species to the Pleistocene era (Feytaud, 1918). With regards to the United Kingdom, our most important *otiorhynchid* pest is the vine weevil, *O.sulcatus* (F.). Other species of *otiorhynchids* with slightly less economic impact are the clay-coloured weevil, *O.singularis* (L.); red-legged weevil, *O.clavipes* (Bonsd.); pig weevil, *O.porcatus* (Herbst.); strawberry root weevils, *O.ovatus* (Gyll.) and the rough strawberry weevil, *O.rugosostriatus* (Goeze) (Anon, 1981; Foster, 1982).

The first reports of damage to greenhouse plants by *O.sulcatus* (vine weevil) were in Germany in 1834 (Smith, 1932), in 1837 in the UK (Westwood, 1937) and in 1831 in the USA. In recent years the vine weevil has become a major pest to commercial strawberry producers, and with an estimated world strawberry production of close to two million tonnes (UK value of £54 m), vine weevil control has significant economic implications.

1.1 Distribution and Host Range

The vine weevil is endemic to the temperate regions of mainland Europe, extending from Northern Italy and reaching the southerly regions of the Baltic States (Figure 1-1). The easterly boundary of their domain extends deep into central Russia (Anon, 1974). The distribution of the vine weevil can be correlated to the mean regional temperature. Stenseth (1987) noted that the Norwegian population of vine weevil was confined to areas where the mean air temperature did not fall below –4°C during the month of January. Although an upper temperature limit has not been quantified in the field, Stenseth (1979) found that pupal development ceased at a constant 27°C.

From its' European origin, the vine weevil has migrated to other continents (Australasia and the Americas). It is believed that this movement is associated with shipments of plants (Smith, 1932). The populations are again found in the temperate regions of these new habitats e.g. eastern and western coasts of the USA and Canada (Figure 1-1; Warner and Negley, 1976). The spread of the vine weevil eastwards was first identified in New-Zealand by Kingsley (1898). It is now found in south eastern Australia and

Tasmania, and more recently (1981) they have been found at a farm in Santo Gun, Shizuoka Prefecture in Japan (Masaki *et al*, 1984). There has also been report of vine weevil damage to plants in Chile (Prado, 1988).

The vine weevil has a very large host range, although it is the selection of an oviposition site by the leaf-eating adult that determines the host for the relatively immobile root-eating larvae. Studies have shown the selective nature of the adult when locating an oviposition site (Smith, 1932; Shanks, 1980; Hanula, 1988). The polyphagous nature of the adult vine weevil has been shown by Smith (1932) who compiled a list of 77 sensitive plant species, to which the USDA added a further 70 species (Warner and Negley, 1976). The recent discovery of vine weevils in Japan led to tests on economically important and indigenous Japanese plants, where it was found that the adult vine weevil would feed on 101 of the 108 species tested (46/49 families, Masaki *et al*, 1984).

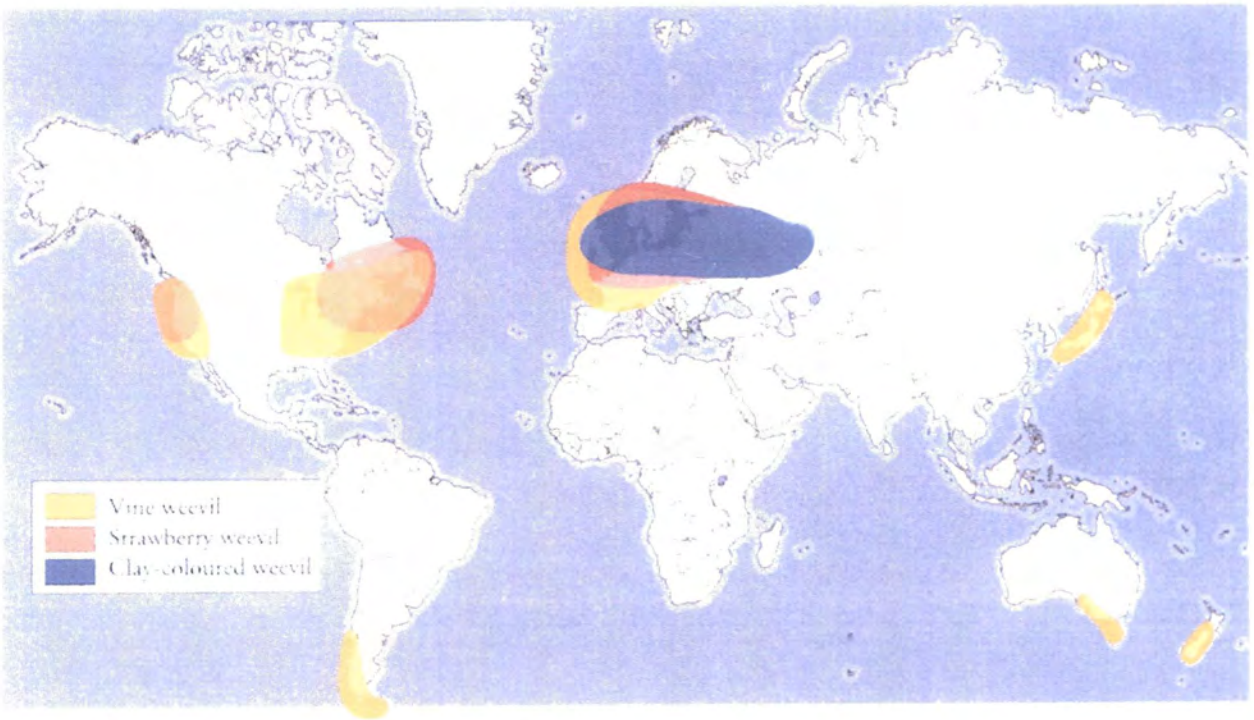


Figure 1-1 Geographic spread of the Vine Weevil, and other economically important *Otiorhynchids*.

1.2 Pest Status

Major vine weevil infestations can result in substantial levels of crop damage, which in turn can cause significant economic losses for the grower. Cyclamen, Taxus and Fragia are especially susceptible to larval root damage. The larval damage threshold has been calculated as a single larva per plant for Cyclamen (Moorhouse, 1992; 1993), three larvae per plant for Rhododendron (La Lone and Clarke 1981) and between two and eight larvae per plant for Strawberry (Penman and Scott, 1976). Neiswander (1953) and Foster (1982) stated that established crops may be more resistant to weevil damage due to their larger root system being able to withstand the effects of feeding. The viability of a plant once infested with vine weevil larvae can be determined by the position of the larvae within the soil (Evenhuis, 1978). One larva can do more damage at the base of the stem than several around the periphery of the root system.

Adult related damage is confined to the leaves of the host plant and manifests as a characteristic 'notching' along the edge of the leaf which is easily distinguishable from damage caused by other insects (Evenhuis, 1978). This adult feeding activity has been associated with the spread of some plant viruses amongst vine yards (Ochs, 1960).

Several modern plant husbandry practices are thought to have been, at least partly, responsible for the increase in the number and frequency of vine weevil infestations. The most significant of these are the widespread use of peat based potting mixes, black polythene mulches and the increased continental trade of container grown plants (Stenseth, 1979; Nielsen and Roth, 1985; Watt *et al*, 1999). Another suggestion is that the extensive and indiscriminate use of insecticides has disrupted the balance between the vine weevil and its natural enemies.

1.3 Life Cycle of the Vine Weevil

The vine weevil life cycle is shown in Figure 1-2. There is one generation per year, but as shown in Figure 1-2 there may be a considerable overlap between the different stages (Schread, 1972) and all stages can be present at the same time (Evenhuis, 1982).

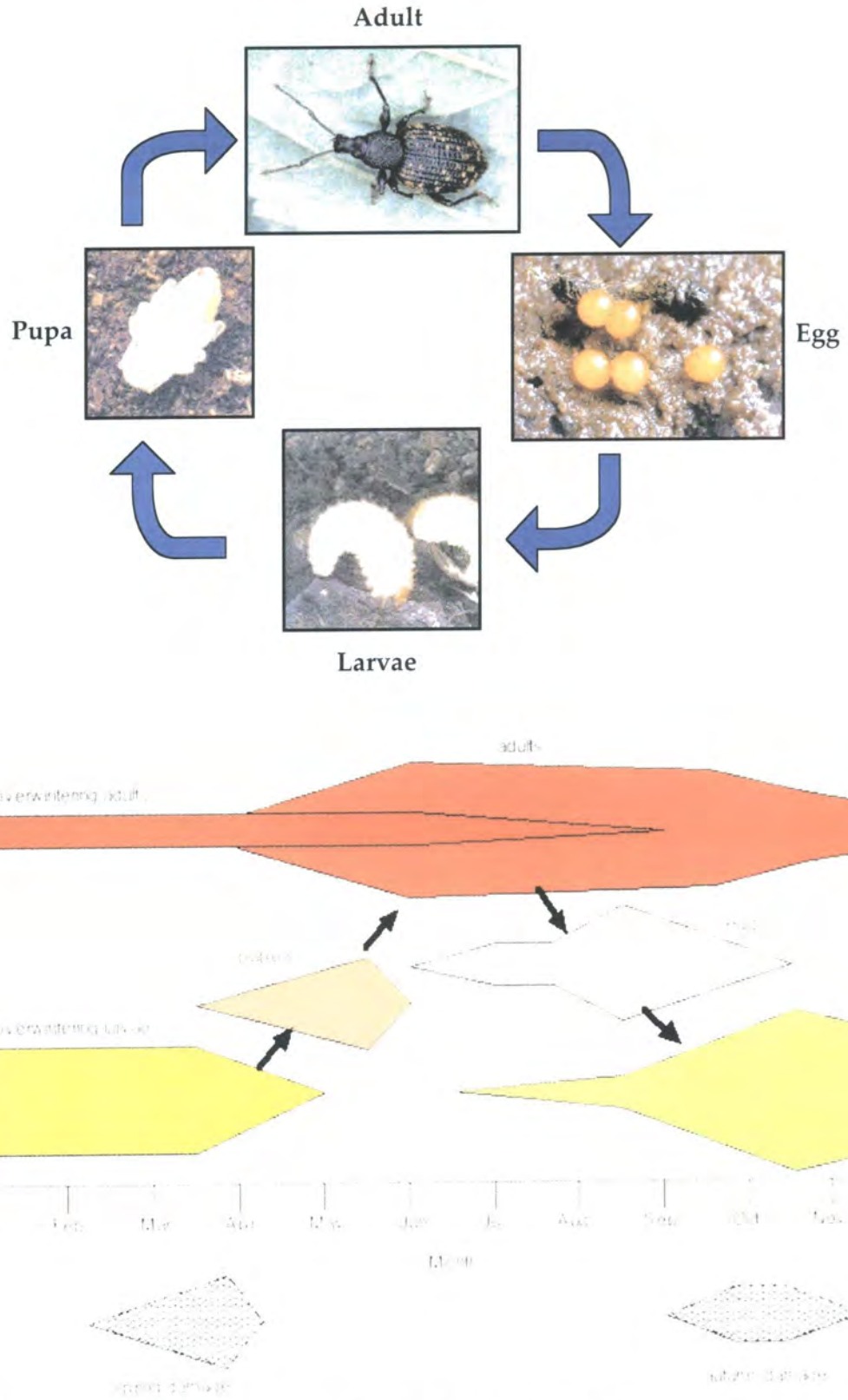


Figure 1-2 Life cycle of the vine weevil and related periods of economic damage (modified from Moorhouse 1992). The diagram shows the duration of each stage and the relative number of individuals present (shown by bar widths) in normal field conditions.

1.3.1 The Egg

Eggs are laid by the adult weevils from June to late September. When first laid the eggs are pearly white, relatively soft and generally spherical with a maximum diameter of 0.8mm (Smith, 1932; Foster, 1982). Flattened or wedge shaped eggs have been observed when laid into cracks or crevices (Smith, 1932). Within 20-80 hours the egg chorion becomes melanated at which point the shell becomes rigid and takes on a chestnut-brown colouration. The process of melanisation protects the egg against fungal infections and environmental extremes (Shanks and Finnigan, 1973). Hatching occurs between 8.4 days at 27°C to 56 days at 9°C (Stenseth, 1979) and the larvae emerge from the shell by cutting and tearing a hole in the shell with their mandibles. Montgomery and Nielson (1979) showed that the eggs could not overwinter in Ohio due to the cold winters, but overwintering may be possible in protected environments.

1.3.2 The Larvae

The larvae are generally white, although body colour is dependent on diet (Moorhouse, 1992). All of the proposed six instars have chestnut-brown heads, which house their strong mandibles, and their entire body is covered with small hairs Figure 1-3. As the larvae age they adopt a typical 'C' posture as a result of the thickening of the thoratic segments (Smith, 1932; May, 1977). The negatively phototrophic larvae are found throughout the plant root zone, and have been found at a depth of several centimetres (Evenhuis, 1978). Both adults and larvae are polyphagous and have been shown to eat the roots of many plant species (Masaki *et al*, 1984), with younger larvae consuming the finer roots and, as they grow older, electing to eat larger membranous roots and even burrowing into the corms, rhizomes or stem bases. With the need to find fresh root material, evidence has shown the vine weevil will travel over 15-20cm to ensure continual development and it is believed that they achieve this by following carbon dioxide gradients within the soil (Klingler, 1957). Egg to pupa development time has been calculated at 198, 130 and 110 days at 12°C, 15°C and 24°C respectively (Stenseth, 1979), and glasshouse results suggest that the vine weevil larvae do not have an obligatory diapause.

Figure 1-3 The soil dwelling larval stage of the vine weevil.

All vine weevil larvae overwinter in small cells formed within the soil at varying depths (15-25cm, Smith, 1932) where they can survive for 150 days at a constant 2°C (7% mortality noted, Stenseth, 1987). During springtime the larvae break free from their overwintering cells, feed voraciously and then pupate within pupal cells at a depth of 10-12cm (Smith, 1932). Smith (1932) noted that once the cell has been formed the larvae voids its' gut contents and becomes a creamy-white colour. This prepupal phase is temperature dependent and may last between 3-34 weeks.

1.3.3 The Pupa

Pupal development, which usually occurs between mid May and mid June in the field, takes between 10 to 50 days at 24°C and 5°C respectively (Schread, 1972; Garth and Shanks, 1978). As the pupa develops, its eyes change from white through brown to black, its wing pads elongate and the body appendages become a rusty-brown colour (Barrett, 1930).

1.3.4 The Adult

The adult vine weevils that may be present throughout the year are all parthenogenetic females bearing unfertilised viable eggs (Figure 1-4). No male vine weevils have ever been found, even with extensive scrutiny of natural populations (Feytaud, 1918). This parthenogenisity is seen in many weevils of the *Curculionidae* family and may be explained by the 'glacial refugium' model where the ancestral males died out during the ice age. When kept in culture pseudocopulation between adults is occasionally observed (Moorhouse, 1990; personal observations). This behaviour was similarly observed with cultures of *O.pupillatus cyclophtalmus* (Sol.) by Pardi (1987) who proposed a link with dominance and crowded conditions.

The new adults emerge from their pupal cells some seven to eight days after transformation (Smith, 1932; Klingler, 1959) at which point their body colour has changed from white to a dell black and their elytra are covered with tufts of yellow hair. Peak emergence occurs at the end of May to late June (Garth and Shanks, 1978), although in colder climates adults continue to emerge well into August (Stenseth, 1976).

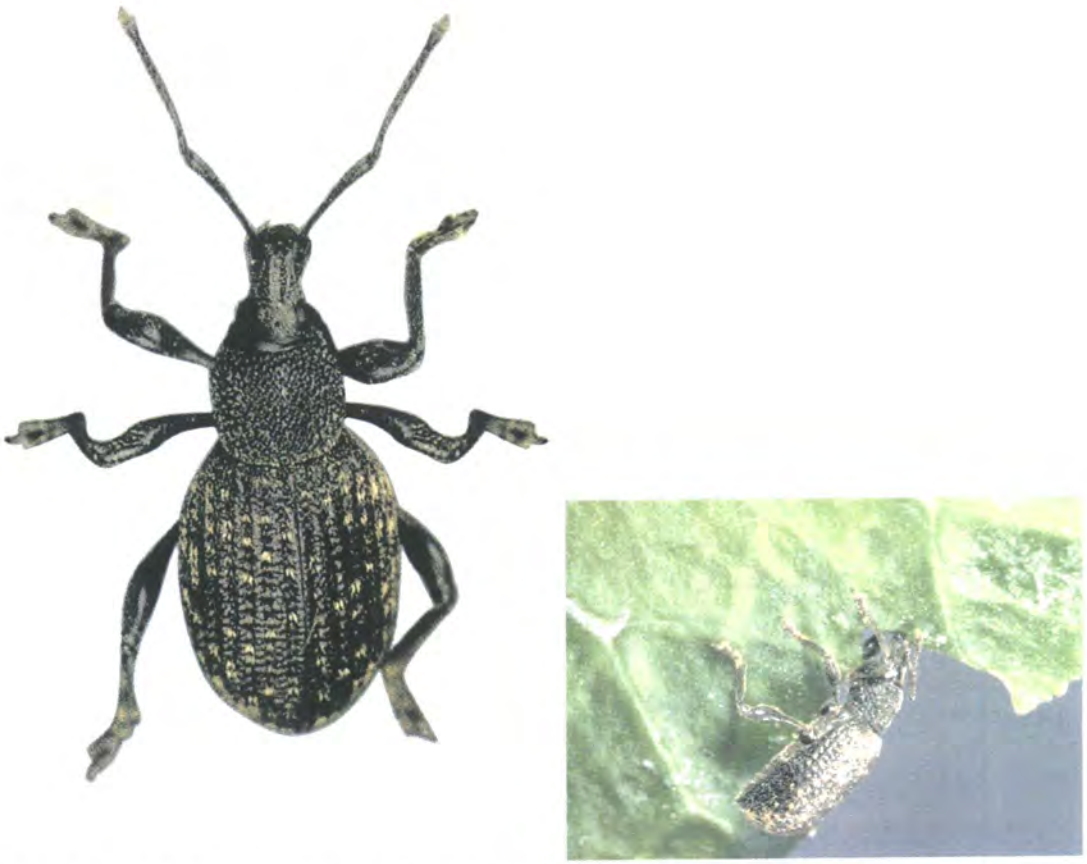


Figure 1-4 The adult vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curclionidae F.).

Figure 1-5 Drawing of a Rhododendron leaf showing typical "notching" damage caused through the feeding of adult vine weevils.

The adult vine weevil is considerably more polyphagous than the larvae and starts feeding on the night following emergence causing the characteristic 'leaf notching', Figure 1-5, on many host plants and weeds. Following several weeks of vigorous feeding the adults begin oviposition. Oviposition which may be influenced by several external factors such as air temperature and relative humidity, with a single adult capable of laying over 1200 eggs. Host preference also governs oviposition. It has been reported that *Taxus cuspidate* is the favoured host (Maier, 1981; Hanula, 1988), and it is believed that the vine weevil detects a chemical signal from the plant through its antennae. Although new adults emerge in late spring many adults survive through the winter, and start oviposition earlier than their younger clones. In sheltered environments the survival rate can be as high as 90% (Evenhuis, 1982), and in controlled laboratory conditions a vine weevil's life span can exceed two years (Moorhouse *et al*, 1992; personal observations)

The elytra of the adult vine weevils are fused, rendering them flightless. This flightless nature significantly limits the migratory capability of the vine weevil. Maier (1978) stated a maximum migratory ability of 80m over a 57 day period, and this migration is restricted to areas of grass with the released weevils not crossing ground covered by weeds. Interestingly it was calculated (Maier, 1978) that populations of vine weevils were spreading across the USA at speeds greater than 15Km $year^{-1}$, this extraordinary speed of migration must therefore be assumed to be a result of the transportation of infested material.

1.4 Natural Enemies

All stages of the vine weevil are subject to predation by a large number of natural enemies. Such examples can be seen in Table 1.1

Infestations by entomogenous fungal species also cause mortality in every stage of the vine weevil (Moorhouse *et al*, 1992), and there are reports of *Bacillus cereus* giving natural control (Marchal, 1977). Recent biological control laboratory experiments have shown that the entomopatogenic nematodes (*Steinernama* and *Heterorhabditis* spp.) give adequate protection against vine weevil larvae (Bedding and Miller, 1981).

Vine Weevil Stage	Predator	Reference
Adult	Hedgehogs	Feytaud, 1918
	Rodents	Moorhouse *et al*, 1992
	Finches etc.	Theobald, 1911
	Reptiles and Amphibians	Feytaud, 1918
	Arachnids	Moorhouse, 1992
Larvae	Carabid and Staphylinid beetles	Feytaud, 1918; Smith, 1932
	Dipteran and Hymeuopteran parasitoids	Moorhouse *et al*, 1992
Eggs	Carabid species	Garth and Shanks, 1978
	Common earwig	
	Ants	Smith, 1932

Table 1.1 Natural predators of each stage of the vine weevil

1.5 Current Vine Weevil control Strategies

1.5.1 Chemical Control

In the decade from 1975 to 1985 the Royal Horticultural Society have encountered a ten fold increase in the number of requests for advice on vine weevil control. This surge in concern over damage caused by the vine weevil and ways of controlling this pest can be explained by the withdrawal of approval by DEFRA for persistent organochlorine insecticides such as DDT and aldrin. The substituted insecticides are not particularly effective against the larvae (Nielsen *et al*, 1975). With regards to the strawberry industry, in which the vine weevil is a major economic pest, organo-phoshorus-based insecticides are used intensively, often as drenches which are applied after dark to kill adult weevils (Anon, 1998). Such compounds include, chloropyrifos used by European growers and aziriphos-methyl or malathion used in North America. Although such practices may reduce adult numbers, larvae remain unaffected and continue to cause damage. The protection of nursery stocks has been enhanced through the use of a slow release granular formulation of chlorpyrifos (SuSCon Green, Crop Care Australasia) or a microencapsulated formulation of fonofos (Watt *et al*, 1999). Although successful on a small scale in the confines of container grown plants these products may not be suitable to control field infestations due to the difficulties of incorporating the precise dose of the insecticide into the soil, and the relatively high costs involved in such a process (Watt *et al*, 1999). Recent trials using fironil (a phenyl pyrazole insecticide), azadirachtin (neem extract) and Gowan Cryolite bait have shown promising levels of control over weevils, and are recommended in Western USA (Anon, 1998). However, none is as effective as aldrin. The effective localisation and targeting of insecticides used against the vine weevil will certainly remain problematic, and will undoubtedly remain unacceptable for fruit spraying close to harvest.

1.5.2 Biological Control

As described earlier the vine weevil is preyed upon by a host of natural enemies, however few of these have been transferred into successful IPM (Integrated Pest Management) schemes. Entomopathogenic nematodes parasitise all larval stages, pupae and newly emerged adults (Bedding and Miller, 1981) in protected cultivation, although they have proved less than reliable under field conditions. The major inhibitory factor when using nematodes is their susceptibility to cold, but several

research centres are currently attempting to isolate strains that are effective at temperatures below 10°C (Steiner, 1996). The use of entomogenous fungi may also aid in the control of the vine weevil, however the high costs involved in registering a microbial bio-control agent in the UK may prove inhibitory.

1.5.3 Other Methods of Control

There have been several alternative plant husbandry techniques suggested to aid vine weevil control which include; using physical barriers (i.e. adhesion strips) to control the flightless weevil; exercising adequate crop rotation; and the removal of fruit foliage after harvest to disrupt oviposition (Garth and Shanks, 1988)

We are now in urgent need of an effective and environmentally sound regime to control the every increasing damage caused by the vine weevil. Failure to do so could result in an estimated loss in excess of 70% of the target crop in second and subsequent years.

1.6 Insect Digestive Enzymes

The ability of insects to populate and exist in many diverse environments may well, in part, be explained by the vast array of digestive enzymes they have to call upon. This bank of enzymes allows insect to exploit a wide range of food sources which include organic materials such as; refractory wood; humus; wool and wax; to more digestible substances such as; micro-organisms; plant and animal tissue; and finally nutrient imbalanced diets such as plant sap (Terra *et al*, 1996). Evidence collected from the characterisation of insect gut enzymes shows that each species draws upon this enzyme bank to ensure they have an optimal digestive system.

Phytophagous insects also have the ability to alter their standard enzyme profile to circumvent the effects of protease inhibitors or plant secondary metabolites (Bown *et al*, 1997; Harborne, 1993).

This section serves as an insight to what may be discovered in the vine weevil gut, and how it coordinates its digestive processes.

1.6.1 Peptidases

Pepidases (peptide hydrolases, EC3.4) are enzymes that hydrolyse peptide bonds, these include the proteases (endopeptidases, EC3.4.21-24) and exopeptidases (EC3.2.4.11-19). Proteases are peptidases acting upon internal peptide bonds of proteins and are classified on the basis of catalytic mechanism. These short peptides are they acted upon by exopeptidases to generate amino acids.

Serine proteases have a serine residue as the catalytic amino acid and include the important enzymes trypsin and chymotrypsin. Cysteine proteases posses a cysteine as the catalytic residue, whereas aspartic proteases depend on a carboxyl residue for activity (Terra *et al*, 1996; Baret *et al*, 1998). The final category of peptidase, metalloprotease, contain a metal ion which is involved in catalysis. This category includes the important digestive exopeptidase carboxypeptidase.

All four classes have been identified as playing roles in protein digestion in different species within the insect kingdom. (Terra 1996)

1.6.1.1 Serine Proteases

The serine proteases; trypsin, chymotrypsin and elastase, which belong to a common superfamily, are responsible for the initial digestion of proteins in higher animals. Both trypsins and chymotrypsins have been found throughout the insect world, with the exception of those species belonging to the *Cucujiformia* family.

The three afore mentioned classes of digestive serine proteases can be identified by their substrate specificity, trypsin specifically cleaving C-terminal to residues carrying a basic side chain (lys, arg), chymotrypsin preferentially cleaving C-terminal to residues carrying a large hydrophobic side chain (phe, tyr, leu), and elastase showing a preference for cleaving C-terminal to residues carrying a small neutral side chain (ala, gly).

Insect serine protease sequences contain typical hydrophobic signalling (secretion) sequences, followed by sequences of 7-40 amino acid residues prior to the sequence of the N-terminus of the mature enzyme. The sequences contain an arginine residue at the putative activation cleavage site. Like mammalian serine proteases it is thought that at

least some insect digestive enzymes are produced as zymogens, and are activated by proteolytic (perhaps tryptic) removal of an N-terminal peptide (Reeck *et al*, 1997). These enzymes generally have Mr values between 20,000 and 35,000, and exhibit optimal activity at high pH .

Chymotrypsins also have high pH optima and most of the examples isolated from insects have Mr values of 20,000-30,000. Unlike trypsins, the chymotrypsins preferentially cleave protein chains on the carboxyl side of aromatic amino acids.

1.6.1.2 Cysteine Proteases

For many years serine proteases were the only insect midgut proteases that were characterized. Nevertheless, after Gooding (1969) suggested that enzymes similar to mammalian cathepsins may be present in insect midgets, cysteine proteases were conclusively demonstrated in *Hemiptera, Heteroptera,* and in *Coleoptera.* In *Coleoptera,* cysteine proteases are known with more details in species of *Bruchidae.* By assaying midgut homogenates in the presence or absence of specific activators and inhibitor, cysteine proteases were also found in the following *Coleoptera* families: *Tenebrionidae, Chrysomelidae, Coccinelidae, Curculionidae, Meloidae* and *Silphidae* (Terra *et al,* 1996). All these families, except for *Siphidae,* belong to the series *Cucujiformia* of *Coleoptera.* The apparent widespread occurrence of midgut cysteine proteases in *Coleoptera* led Murdock *et al* (1987) to suggest that these enzymes are commonly used as digestive enzymes in *Coleoptera.*

1.6.1.3 Aspartic Proteases

Aspartic proteases have been identified in several families of *Coleoptera* (Wolfson and Murdock, 1990; Silva and Xavier-Filho, 1991), the sequences of which have been shown to be similar to the cathepsin-D proteases found in vertebrates. These proteases exhibit optimal activity under acid conditions and they hydrolyse internal peptide bonds of protein chains.

1.6.2 Glycosidases

The group of enzyme known as glycosidases degrade oligo- and polysaccharides. They can be divided into two categories. The first group termed depolymerises, cleave

internal bonds of polysaccharides. These enzymes are named after their substrate and are referred to as amylases, cellulases, pectinases and chitinases. The second group contains those enzymes that hydrolyse oligo- and disaccharides.

1.6.2.1 α-Amylase

α-Amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) constitute a family of endo-amylases that catalyse the hydrolysis of α-D-(1,4)-glucan linkages in starch components, glycogen and other carbohydrates. The enzyme plays a key role in carbohydrate metabolism of microorganisms, plants and animals. Moreover, several insects, especially those similar to the seed weevils that feed on starchy seeds during larval and/or adult stages, depend on their α-amylases for survival. Research on starch digestion as a target for control of starch-dependent insects was stimulated when results showed that α-amylase inhibitors from *Phaseolus vulgaris* seeds are detrimental to the development of cowpea weevil *Callosobruchus maculates* and Azuki bean weevil *Callosobruchus chinensis* (Ishimoto and Kitamura, 1988; 1989).

The carbohydrate digestion of bruchid weevils, such as the Mexican bean weevil *Zabrotes subfasciatus* and the cowpea weevil *C. maculatus*, occurs mainly in the lumen of the midgut. High enzymatic activities against starch, maltose, maltodextrins and galactosyl oligosaccharides were found in the luminal fluid, while only aminopeptidase activity was predominantly associated with gut membrane

1.6.2.2 Cellulases

Until recently it was presumed that cellulose-degradation was the sole domain of microbes and plants, and that animals which fed on cellulose rich diets required the assistance of cellulolytic gut symbionts. This is surprising when one considers the nutritionally poor diets of many insect species, i.e. termites and other wood-eating insects.

Evidence is currently appearing that suggests that insects can themselves generate cellulolytic enzymes and benefit directly from the digestion of this abundant polysaccharide.

Most cellulolytic enzymes consist of distinct catalytic and cellulose-binding domains (CBDs). This domain structure is seen in many enzymes that degrade other insoluble carbohydrates such as raw starch and chitin. It is presumed that such binding domains improve the binding and facilitate the activity of the catalytic domain on the insoluble but not on soluble substrates (Linder and Teeri, 1997).

Cellulases can be assigned to one of two different groups of enzyme depending upon their mode of action: the endoglucanases which cleave the cellulose glucan chains at interior sites and the cellobiohydrolases which act processively to release cellobiose primarily from the chain ends. Both type of enzyme cleave ß-1,4-glycosidic bonds and their endo/exo specificity seems to be determined by the conformation of the active site region. While the endoglucanases have open active site clefts, the exoglucanase active sites are located in tunnels formed by long loops in the protein structure (Henrissat *et al*, 1989).

Within the microbial world cellulases have been identified as part of a macromolecular machine termed a cellulosome, whose components interact synergistically to catalyse the efficient degradation of cellulose. The cellulosome is bound to the cell surface and each enzymic component is tethered by specialised scaffoldin subunits (Bhat and Bhat, 1997). Currently no evidence for a similar occurrence exists in the insect world.

1.6.2.3 Pectic Enzymes

Pectin is the major component of the middle lamella of plant cell walls (Jarvis, 1984). Chemically, pectin is a polymer comprised mainly of methylated galacturonic acid units (Van Buren, 1991). Several different classes of enzymes, collectively called pectinases, participate in the breakdown of pectin. Pectinesterase (EC 3.1.1.11) catalyzes the hydrolysis of methyl ester groups in pectin, releasing methanol and converting pectin to pectate (the unesterified polymer). Because the pectin degrading enzymes polygalacturonase and pectate lyase can efficiently attack only de-esterified pectin, pectinesterase plays an important role as the first step in the breakdown of pectin (Collmer *et al*, 1988). Pectin methylesterases have been studied extensively from microorganisms and plants (e.g., Collmer and Keen, 1986; Pressey and Woods, 1992; Rillo *et al*, 1992; Lim and Chung, 1993; Barras *et al*, 1994; Glover and Brady, 1994; Christgau *et al*, 1996). In addition, numerous insect species have been reported to have pectinase activities (Adams and McAllan, 1956, 1958; King, 1972; Laurema and

Nuorteva, 1961; Ma *et al*, 1990; Shukle *et al*, 1985). The functions of pectinases in insects are not well understood, partly because few pectinase enzymes have been purified from insect, and currently none of the genes encoding for these enzymes have been cloned from an insect origin.

It has been suggested that pectinases, in salivary secretions, may be involved in facilitating the penetration of the mouthparts of piercing-sucking insects such as aphids into host plants (Adams and McAllan, 1958; Ma *et al*, 1990). Dreyer and Campbell (1987) pointed out that the quality and quantity of pectin in host plants can affect the capability of insects to attack host plants, and suggested that the aphid pectinases may be involved in the development of aphid biotypes and host-plant resistance through the levels of enzyme activity and levels of methylation of pectin.

The rice weevil, Sitophilus oryzae (L.), has been reported to have high polygalacturonase activity (Campbell *et al*, 1992; Campbell, 1989). It was from rice weevil that Shen *et al* (1996) were able to be the first to purify an insect polygalacturonase. Recently, polygalacturonase has been purified from another coleopteran species (Doostdar *et al*, 1997)

1.6.2.4 Chitinases

The structural polysaccharide chitin found in insects and fungi is a linear homopolymer of 2-acetamido-2-deoxy-D-glucopyranoside (N-acetyl-glucosamine, GlcNAc) connected by ß-1,4-linkages. Chitinases cleave the chitin substrate forming shorter chains of GlcNAc. Chitinases have been identified in the guts of many orders of insect (Vonk and Western, 1984), however its role as a true digestive enzyme is not clear due to its known house-keeping role.

The degradation of cuticular chitin by chitinases is a vital step in the life cycle of insects because insects undergo periodic ecdysis and metamorphosis. The unsclerotized layers of the cuticular exoskeleton should be digested prior to ecdysis and metamorphosis. During the molting process, chitin in the insect cuticle is degraded by chitinolytic enzymes. These enzymes have been purified and characterized from various insect species, including *Manduca sexta* and *Bombyx mori* (Kramer *et al*, 1985; Kramer and Koga, 1986; Koga *et al*, 1989, 1992). The insoluble chitin is first cleaved into oligosaccharides by the endosplitting chitinases and then the soluble oligosaccharides

are further hydrolyzed to 2-acetylamido-2-deoxyglucopyranoside by the exo-type chitinolytic enzyme, ß-*N*-acetyl-glucosaminidase (Fukamizo and Kramer, 1985). The former reaction is a rate-limiting step for the chitin degradation (Fukamizo and Kramer, 1987). A gene encoding the chitinase of *M. sexta* has been cloned and its expression during metamorphosis explored (Kramer *et al*, 1993). Kramer *et al* (1993) reported that the chitinase gene of *M. sexta* was expressed in epidermal and gut tissues during the larval-pupal transformation and its transcription was stimulated by the morphogenetic hormone 20-hydroxyecdysone and inhibited by the juvenile hormone mimic, fenoxycarb (Kramer *et al*, 1993). The cloning and characterization of chitinase genes of insects may provide a clue for the utilization of these enzymes as a control agent of insect pests.

1.6.2.5 ß-Glucosidase and ß-Galactosidase

ß-Glucosidases catalyse the hydrolysis of terminal, non-reducing ß-1,4-linked monosaccharide residues from the corresponding glycoside. The enzyme nomenclature is dependant upon the monosaccharide removed from the sugar e.g. glucosidase (glucose), galactosidase (galactose).

These hydrolases can be classified into two groups based upon their activities; glycosyl ß-glycosidase, which are active against di- or oligosaccharides such as cellobiose and lactose, and aryl (or alkyl) ß-glycosidases that are active upon a monosaccharide linked to a non-sugar residue (aglycone) such as *p*-nitrophenyl-ß-D-glucoside (NPßGlu). Those ß-glucosidases found in insects are divided into three classes as defined by their substrate specificity to either one or both of the above categories.

1.7 Defensive Proteins of Plants and their role in Conferring Insect Resistance

The use of transgenic plant technology to produce crops resistant to insect attack, via the introduction of novel resistance genes, is establishing its value as an integral component of a crop management programme. Genes encoding insecticidal proteins have been isolated and characterised from several plant species, and the insecticidal properties have been transformed into novel crops through genetic engineering.

1.7.1 Protease Inhibitors

Protease inhibitors have been identified in many plant species through sequence homology, and have been shown to be synthesised and accumulate in storage tissue, both in seeds and in vegetative storage tissue such as potato tubers. Levels of the protease inhibitors have been identified as high as 2% of total protein and have been shown to provide a protective role against insect herbivory.

Ryan *et al* (1972) demonstrated that in certain solanaceous plants protease inhibitors were induced by insect feeding or mechanical wounding. It was also shown that the induction of protease inhibitors occurred throughout the wounded plant by means of a protease inhibitor-inducing factor (PIIF). PIIF released at the site of the wound is translocated to other leaves where it initiates the synthesis and accumulation of inhibitors (Ryan, 1972).

The majority of protease inhibitors are products of multigene families (Watt *et al*, 1999), they are relatively small proteins with molecular weights of between 5 kDa and 25 kDa (Ryan, 1990), and they act in direct competition with the native substrate of the protease (Laskowski and Kato, 1980). The small molecular weight of protease inhibitors may be advantageous in allowing them to pass freely through the phloem sieve plates to populate the whole plant.

The effects of ingesting protease inhibitors on digestion may be overcome by synthesizing increased amounts of proteases. This however in mammals this causes pancreatic hypertrophy, leading to loss of body weight and even death (Broadway and Duffey, 1986).

There are many reports in the literature about protease inhibitors and their effects on insect growth and development. To date the majority of research centres are focusing upon serine and cysteine protease inhibitors which have been shown to be important in defence against attack. The effects of inhibitors of metallo and aspartyl proteases on insects and their gut enzymes have been little studied although an inhibitor of aspartyl proteases has been reported in potato (Ritonja *et al*, 1990) and inhibitors of carboxypeptidase-A (metallo protease) have been isolated from potato and tomato (Rancour & Ryan, 1968; Hass & Ryan, 1980).

The potential use of proteinaceous enzyme inhibitors as protective agents in crop production through the use of transgenic plants is becoming increasingly favourable as research progresses. However progress towards understanding the precise nature of interactions between inhibitors and insect digestive enzymes in different species is necessary. Additional problems due to insect adaptation or resistance are likely to develop in the field. Several insect species have been reported to adapt to protease inhibitors by altering their complement of secreted proteases such that insensitive enzymes are predominant (Broadway, 1995; Jongsma *et al*, 1995; Boulter & Jongsma, 1995). Adaptation has been shown to be influenced by host range with polyphagous insects better adapted to tolerate non-host protease inhibitors than more specialised feeders (Broadway & Villani, 1995). That adaptation through the induced production of inhibitor insensitive protease activity may be due to variants of existing enzymes rather than due to the induced activity of enzymes of different mechanistic classes has also been shown (Bown *et al*, 1997). Bown *et al* (1997) have suggested that multiple, varying protease encoding genes may represent an adaptive mechanism whereby insects can reduce the deleterious effects of plant protease inhibitors. Further studies in this area are required if adaptation and resistance to protease inhibitors in the field is to be managed successfully.

1.7.1.1 Inhibitors of Serine Proteases

As described earlier serine protease inhibitors have been isolated from several species of plant especially from storage tissue such as winged bean seeds (Giri *et al* 2003), these inhibitors inhibit endopeptidases such as trypsin and chymotrypsin such as those found in many *Lepidopteran* pests but have no distinguishable roll in the regulation of the plants own protease activity (Gatehouse *et al*, 2000).

A common mechanism of serine protease inhibitors is the direct inhibition of digestive proteolytic enzymes whereby the inhibitor directly mimics the normal substrate for the enzyme and prevents the completion of normal peptide bond cleavage (Felton & Gatehouse, 1996). Many of these inhibitors are capable of simultaneously inhibiting two molecules of enzyme per inhibitor molecule (Gatehouse & Hilder, 1994). It is possible that a situation analogous to protease inhibitor effects in mammals exists in insects. In mammals major effects are the loss of nutrients through pancreatic hypertrophy and the overproduction of digestive enzymes (Liener, 1980). Comparatively little is known as yet about hormonal control and the synthesis/secretion of digestive proteases in insects. Enzyme secretion is thought to be largely constitutive in continuous feeders. However, virtually all insects studied show considerable changes in digestive enzyme levels during the digestion process and this is indicative of regulated secretion (Lehane *et al*, 1996). Paracrine and/or prandial (i.e. the direct interaction of an element of the meal with digestive-enzyme producing cells) mechanisms are thought to be the main factors controlling enzyme synthesis and secretion (Lehane, 1995). However, distinguishing between the two probably interdependent mechanisms and the role of possible direct and indirect hormonal influences presents considerable difficulties. Thus details of the mechanisms involved remain to be established. Various studies have demonstrated that the insect's physiological response to a diet containing enzyme inhibitors is complex and involves feedback mechanisms that determine and adjust enzyme levels in the gut (Lehane *et al*, 1996). The chronic ingestion of protease inhibitors results in the hyperproduction of proteolytic enzymes which, by limiting essential amino acids for protein synthesis, manifests as a reduction in growth and development (Broadway & Duffoy, 1986). Accordingly the supplementation of methionine in the diet of *C.maculatus* has been shown to overcome the anti-nutritional effects of the cowpea trypsin inhibitor (CpTI) (Gatehouse & Boulter, 1983).

Over-expression of protease inhibitors in transgenic plants has been explored as a strategy for inferring insect resistance. CpTI, a Bowman-Birk serine protease inhibitor, shows no toxicity to mammals at levels of 10 % total protein (Pusztai *et al*, 1992) and has been shown to be effective against a wide range of economically important field and storage pests. The CpTI gene was the first of plant origin to be transferred successfully to another plant species resulting in enhanced insect resistance (Hilder *et al*, 1987). Tobacco plants expressing CpTI at levels of up to 1 % of total soluble proteins were shown to have enhanced resistance to *H. virescens*, a serious pest of tobacco, cotton and maize. Significant protection afforded by CpTI was subsequently demonstrated for

other Lepidopteran pests including *H. zea, Spodoptera littoralis* (Biosd) and *M. sexta* (Joh.). Significant protection of CpTI expressing tobacco plants against *H. zea* has also been shown in field trials (Hoffman *et al,* 1991). Different CpTI expressing crops including potato, oil seed rape, rice and soft fruit have now been produced. CpTI transgenic rice has exhibited enhanced levels of protection against 2 major rice insect pests (*Sesamia inferens* and *Chilo suppressalis*) (Xu *et al,* 1996). Significant resistance to the vine weevil (*Otiorhynchus sulcatus*) has been observed in CpTI transgenic strawberry plants (Graham *et al,* 1995; 1997; 2002). In addition to the CpTI protease inhibitor, a Kunitz trypsin inhibitor (SKTI) from soybean has shown to confer resistance in transgenic sugarcane against *Diatraea saccharalis* (Falco and Silva, 2003)

Transgenic plants with genes for other plant protease inhibitors have also been tested for their insecticidal activity. The tomato inhibitor II gene (PI-II), which encodes a trypsin inhibitor with some chymotrypsin activity, when expressed in tobacco was shown to provide protection against *M. sexta.* Levels of expression were correlated with larval weight reduction (Johnson *et al,* 1989). However, tobacco plants expressing tomato inhibitor I at similar levels had no detrimental effects upon larval development (Johnson et al., 1989). Carbonera *et al* (1992) reported increased mortalities of the blackcutworm *A. ipsilon* and armyworm *S.littoralis* exposed to plants transformed by a barley trypsin inhibitor. Experiments where the gene encoding the potato inhibitor II (which inhibits chymotrypsin) was engineered into tobacco showed that larvae of the green looper (*Chrysodeixis enosmia*) exhibited a significant reduction in growth on transgenic leaf tissue (Mcmanus *et al,* 1994). Popular trees have been transformed with the coding region of potato protease inhibitor II (PIN2), and expression of the transgene was detected by enzyme-linked immunosorbent assays (ELISAs) and western analysis (Klopfenstein *et al,* 1993). During bioassays using these trees against the willow leaf beetle Klopfenstein noted a significant difference from the untransformed clone leaf consumed in one transgenic line.

Further examples of the use of serine protease inhibitors in transgenic crop protection has been shown by the use of protease inhibitors can synergise the insecticidal activity of Bt toxins has been indicated by work carried out by Zhao *et al* (1996) who expressed 2 insecticidal genes in the same transgenic tobacco plant. One encoded CpTI and the other a toxin gene from *B.thuringiensis*. Plants that contained both genes were more resistant to *H. armigera* larvae compared with plants containing either of the individual genes.

1.7.1.2 Cysteine Protease Inhibitors

Most cysteine protease inhibitors belong to a family of proteins known as cystatins. Within the family there are three distinct forms of cystatin; Type I, Type II, and Type III (Abe *et al*, 1987), all containing a Gln-Val-Val-Ala-Gly consensus sequence which is likely to be involved in the inhibitory function of these proteins (Hirado *et al*, 1985).

Type I cysteine protease inhibitors, termed stefins, are small single-chain proteins of about 11kDa, that lack disulphide bonds and are not glycosylated (Barrett, 1986). Type II cystatins are slightly larger than the stefins with molecular weights of about 13kDa. Their main distinguishing characteristic is the presence of two disulphide bonds near the carboxyl-terminus (Turk and Bode, 1991) and a typical lack of glycosylation. The final member of the family is the Type III cysteine protease inhibitor (Kininogens), which can be divided into a further three types; H-Kininogen, (M_r of about 120kDa); L-Kininogen, (M_r of about 68kDa); and T-Kininogen (major acute phase protein), (M_r of about 68kDa). All kininogens are single-chain peptides, which share a common amino-terminus and exhibit variability at the carboxyl-terminus (Turk and Bode, 1991).

Several studies have demonstrated *in vitro* inhibition of insect digestive proteases by cysteine protease inhibitors (Liangetal., 1991;Hinesetal., 1991;Gillikinetal., 1992; Michaud *et al*, 1993, Schuler *et al* 1998). A Type II cysteine protease inhibitor isolated from rice, oryzacystatin (Abe *et al*, 1987), has been extensively studied and shown in artificial diet studies to inhibit the digestive proteases of *Sitiophilus oryzae* (Liang *et al*, 1991) and *Diabrotica undecimpunctata howardi* (Edmonds, 1996). Few studies have been reported of their insecticidal effects *in planta* although the oryzacystatin gene has been engineered into poplar trees for resistance towards *Chrysomela tremulae* F. (Leple *et al*, 1995). Zaplachinski *et al* (1997) expressed large quantities of OC-1 in seedling of *B.napus* which conferred resistance against flea beetle larvae.

1.7.2 α-Amylase Inhibitors

A number of studies of inhibitors of enzymes other than proteases, mainly inhibitors of α-amylase, have been carried out. α-Amylase inhibitors have been purified from many plants and are especially abundant in cereal grains (Garcia-Olmedo *et al*, 1987). As wound induced synthesis of α-amylase inhibitors by insect attack has not been observed their role as defensive proteins remains speculative. Indirect evidence for a

protective role is provided by the common bean (*Phaseolus vulgaris*) α-amylase inhibitor αAI which inhibits the activity of certain mammalian and insect α-amylases but not that of plant enzymes (Chrispeels, 1997).

The first αAI sequence was identified (Moreno and Chrispeels, 1989) on the basis of amino acid sequence identity of a purified heat stable αAI and a previously cloned cDNA referred to as 'bean lectin' based on its similarity to PHA (Hoffman *et al*, 1982). This inhibitor is referred to as αAI-1. The common bean has long been known to be toxic to mammals and to contain factors that protect it against certain species of bruchid. The genes for 3 common bean defence proteins, namely phytohaemagglutinin (PHA), arcelin and αAI are encoded at a single locus in the *P. vulgaris* genome (Nodari *et al*, 1993) and it is thought that the homologous genes have arisen by duplication of an ancestral gene. αAI has been found in most domesticated bean varieties and in wild accessions (Chrispeels, 1997). Bean αAI and wheat α-amylase inhibitors have been shown to have a variable effect on the activity of amylases of different origin (Ishimoto and Kitamura, 1988; Gatehouse *et al*, 1986). The α-amylase of the cowpea weevil is strongly inhibited by bean αAI and this bruchid does not attack beans, whereas the α-amylase of the Mexican bean weevil which is a major pest of beans is inhibited only weakly (Ishimoto and Kitamura, 1989). Subsequent studies have established that this lack of inhibition is caused by digestion αAI by gut proteases (Ishimoto and Kitamura 1992; Ishimoto and Chrispeels, 1996). αAI is thus thought to be a major factor in the resistance of beans to the cowpea weevil. The αAI protein has no inhibitory activity until it is proteolytically processed at Asn 77 (Puego *et al*, 1993). It is thought that proteolytic processing may bring about a conformational change that creates an active site allowing αAI to bind to its target enzyme (Chrispeels, 1997). The residues -Try-Gln-Trp-Ser-Try- have been shown by mutational analysis to be involved in the active site of the inhibitor (Chrispeels, 1997). Complex formation has a pH optimum of around 5.5 and therefore αAI inhibits amylases in the acid midgut of Coleoptera, but not in the alkaline midgut of Lepidoptera. For this reason αAI is well suited for defence against bruchids, which are dependent on starch digestion for carbon nutrition.

Expression of the αAI-1 cDNA in tobacco seeds confirmed that the sequence encoded an active inhibitor (Attabella and Chrispeels, 1990). Transgenic seeds exhibited antigenicity to bean α-amylase inhibitors and the expression of polypeptides of molecular weight corresponding to these particular inhibitors was shown. Seed extracts were found to be active against porcine pancreatic a-amylase and α-amylase present in

the midguts of the meal worm *Tenebrio molitor* L. The potential for use of this gene to protect leguminous seeds from Coleopteran pests is being investigated. Shade *et al* (1994) produced αAI bean expressing pea plants. Transgenic α-AI had a significant impact upon the azuki bean weevil larval survival. This larval mortality was closely correlated with the biochemical detection of αAI-1 in individual seeds. The response of the cowpea weevil was more variable although complete insect mortality was recorded for α-AI levels of O.8-1.0 %. Schroeder *et al* (1995) have since shown that the α-AI expressing pea seeds significantly inhibit the development of the pea weevil (*Bruchus pisorum*), an insect that damages peas in the field. It is likely that protection to other starchy grain legumes that suffer bruchid attack could be similarly afforded by the α-AI gene. At present the major constraint upon progress is the lack of suitable transformation methods for species such as cowpeas, mungbeans and chickpeas. Because αAI inactivates intraduodenal amylase in humans (Layer *et al*, 1985) it is likely to be an antinutritional factor that must be heat denatured prior to consumption.

Many varieties of bean have been shown to contain 2 αAI inhibitors (Iguti & Lajolo, 1991), indicating that αAI, like the homologous protein PHA, may be encoded by 2 different genes (Chrispeels, 1997). αAI-2 showing 75 % amino acid sequence identity with αAI-1 has been purified from a wild accession of the common bean resistant to Mexican bean weevils (Mirkov *et al*, 1994; Suzuki *et al*, 1994). Unlike αAI-1, it is an effective inhibitor of Mexican bean weevil α-amylase (Suzuki *et al*, 1993). The inhibition of Mexican bean weevil a-amylase aAI-2 expressed *in planta* has not yet been successfully demonstrated (Mirkov *et al*, 1994).

1.7.3 Lectins

1.7.3.1 Plant lectins; definition, classification and distribution.

Plant lectins are a very large and heterogenous group of proteins most recently defined as proteins which reversibly bind specific mono- or oligosaccharides and possess at least one sugar binding domain (Peumans & Van Damme, 1995). Most lectins contain multiple sugar binding sites and can therefore give rise to the formation of cell aggregates, by cross-linking carbohydrate side chains of cell surface glycoproteins. The presence of lectins with a solvent are usually assayed by visual agglutination of erythrocytes. The term haemagglutinin, still used as a synonym for lectin, dates back to 1888 when Stillmark observed that preparations from castor bean extracts agglutinated

red blood cells. Haemagglutinin was replaced in 1948 by the term lectin (from the Latin verb 'legere', meaning to select) following the discovery of the blood group specificity of certain agglutinins (Renkonnen, 1948; Boyd & Regrera, 1949). Accordingly an earlier definition of lectins as carbohydrate-binding proteins of non-immune origin which agglutinate cells or precipitate glycoconjugates was based primarily on the sugar specificity and inhibition of agglutination reaction (Goldstein, 1980). However, this definition excluded poorly agglutinating toxins known to contain lectin subunits (e.g. ricin) and those lectins that contain a second type of binding site interactive with non-carbohydrate ligands. Lectins were classically sub-divided into glucose/mannose-specific; galactose/ N-acetylgalactosamine (Gal/GalNAc)-specific; Nacetylglucosamine (GlcNAc)-specific; fucose-specific and sialic acid-specific (Goldstein *et al*, 1986). This however, excludes many lectins such as the strictly mannose-specific snowdrop (*Galanthus nivalis* agglutinin) (GNA) lectin; those that bind only to oligosaccharides and it does not account for the higher affinity of many lectins for oligosaccharides containing 2 or more monosaccharide units.

The most recent definition of lectins is based upon the presence of functionally active carbohydrate binding domains and includes proteins and plant enzymes such as Class I chitinases, and Type-2 ribosome inactivating proteins (RIP e.g. ricin and abrin) which contain, respectively, an N-terminal chitin binding and catalytic domain (Collinge *et al*, 1993) and an N-terminal toxic A chain and a C-terminal carbohydrate binding B-chain (Barbiera *et al*, 1993). Also included are carbohydrate-binding proteins that possess only one binding site and are therefore unable to precipitate glycoconjugates or agglutinate cells such as the mannose binding protein from orchids (Van Damme *et al*, 1994 a,b). Lectin-relatedproteins, evolutionarily and structurally related to lectins but devoid of carbohydrate-binding activity (e.g. arcelins and α-amylase inhibitor from *Phaseolus* spp.) are also included (Mirkov etal., 1994).

Thus 4 major types of lectins, namely, merolectins, hololectins, chimerolectins, and superlectins, are distinguished on the basis of the overall structure of the lectin subunits (Van Damme *et al*, 1998).

Lectins have been isolated from a wide variety of species encompassing almost every major taxonomical classification of flowering plants, and are also present in a variety of non-flowering and lower plants (Etzler, 1985). Whilst lectins are found in virtually all vegetative tissues many plants accumulate lectins in storage tissue such as seeds, bulbs

and bark. Lectins typically account for 0.1-5 % of total seed protein but can reach levels of up to 50 % as exemplified by *Phaseolus* species. Non-seed lectins are also found at levels of 0.1-5 % of total proteins although values of >50 % have been reported for lectins in vegetative storage tissues of some species such as garlic cloves (Smeets *et al*, 1997).

Legume lectins, most commonly found in seeds of the Leguminoseae family, differ considerably in their carbohydrate-binding specificity. Distinguished by their preferential binding to monosaccharides most are specific for mannose/glucose (e.g. *Canavalia ensiformis* lectin (Con A) or gal/GalNAc (e.g. *Arachis hypogaea* lectin). Lectins that interact exclusively with oligosaccharides (e.g. *Phaseolus vulgaris* (PHA) lectin) are also found. All legume lectins contain divalent cations (at specific metal binding sites) essential for their carbohydrate binding activity (Van Damme *et al*, 1998). Legume lectins are synthesised as pre-proteins or pre-pro-proteins on the endoplasmic reticulum (ER), and are converted into mature lectins after post-translational modifications both in the ER and during transport via the golgi system to their final destination, typically vacuoles or vacuole derived organelles. Several legume lectins have been studied by x-ray crystallography (e.g. Con A) (Ronge *et al*, 1991). All lectins, irrespective of their sugar binding specificity, appear to be composed of monomers with a similar 3-dimensional structure, typified by the occurrence of anti-parallel pleated sheets (ß structures). The majority of residues not involved in the ß structures are located in loops and turns interconnecting the strands of the pleated sheets. $Mn2^{+}$ and $Ca2^{+}$ binding sites are highly conserved. Less conserved are amino acids in the carbohydrate binding sites, where substitutions are thought to have facilitated evolution towards proteins with different sugar-binding specificity (Van Damme *et al*, 1998).

Chitin-binding lectins, composed of one or more hevein domains (Raikhel *et al*, 1993), have been isolated from several taxanomically unrelated families, including *Gramineae, Urticaeae, Solanaceae* and *Euphorbiaceae*. In addition to binding chitin, most of these lectins also react with GlcNAc, GlcNAc-oligomers and *N*-acetyl-D-neuraminic acid. Hololectins (true agglutinins), merolectins (monovalent and therefore non-agglutinating) and an extended group of chimerolectins, the Class I chitinases (hevein domain tandemly arrayed with a catalytic domain with chitinase activity) are found within this group.

Since the isolation of GNA by Van Damme *et al* (1987) structurally and evolutionary related lectins have been isolated from 5 different families, namely, *Amaryllidaceae, Alliceae, Araceae, Orchidaceae,* and *Liliaceae*. Due to their unique specificity towards mannose and exclusive occurrence in monocotyledonous species they are classed as monocot mannose-binding lectins. This single superfamily exhibits marked sequence homology (Van Damme *et al,* 1991; 1992; 1994; 1995; 1996; Koike *et al,* 1998). All monocot mannose-binding lectins are composed of polypeptides of approximately 12 kDa. Important differences in processing and post-translational modifications have been identified (Van Damme *et al,* 1998). Most of the lectins are composed of 2 or 4 identical subunits of approx 12 kDa, synthesised as separate polypeptides. These homomeric lectins are synthesised as pre-pro-proteins and converted by co-translational cleavage of a signal peptide and post-translational removal of a C-terminal peptide, to a typically non-glycosylated mature lectin polypeptide (Van Darnme *et al,* 1991; 1992; 1994). There are also several heteromeric forms. Heterodimers, built up of 2 different (but highly homologous) subunits (approx 12 kDa), may be derived from separate preproproteins and processed similarly to homomeric precursors (e.g. *Allium ursinum* lectin (Smeets *et al,* 1994)). A second type are heterodimers or heterotetramers composed of 2 different types of subunit derived from a single precursor with 2 distinct lectin domains such as the heterodimeric garlic (*A. sativam*) lectin ASA-I (Van Damme *et al,* 1992).

Type-2 ribosome-inactivating proteins (RIP) possess a highly specific rRNA N-glycosidase activity and are capable of catalytically inactivating eukaryotic (and in some cases prokaryotic) ribosomes. Type-1 and Type-2 RIPs differ in their subunit composition. Type-1 RIPs are monomeric proteins consisting of a single catalytically active subunit (approx 30 kDa) and as such are not members of the lectin family. Type-2 RIPs are composed of 1,2, or 4 identical subunits. Each subunit consists of 2 structurally and functionally different polypeptides called A and B chains that are covalently linked by a disulphide bond. The A chain has N-glycosidase activity and sequence homology to Type-1 RIPs. The B chain is catalytically active and exhibits carbohydrate-binding activity. Both A and B chains are synthesised as large precursors containing 2 functional domains. Precursors are processed via the excision of a peptide linking the C-terminus of the first domain and the N-terminus of the second domain. This group, like the legume lectins, contains lectins with differing sugar-binding properties although most Type-2 RIPs are specific for gal or GlcNAc. Type-2 RIPs, found in members of taxonomically unrelated plant families, are considered as a superfamily of structurally related lectins (Van Damme *et al,* 1998).

1.7.3.2 Lectins active against Coleoptera.

An involvement of lectins in insect resistance was first proposed by Janzen *et al* (1976) who showed that the PHA was toxic to developing larvae of the bruchid beetle *C.maculatus*. This result was subsequently confirmed by Gatehouse *et al* (1984) although a highly purified lectin preparation was found to be less effective than a crude preparation in retarding insect development. The contaminating protein with insecticidal activity was shown to be αAI, the α-amylase inhibitor (Huessing *et al*, 1991). Other lectins toxic to *C.maculatus*, a major storage pest of cowpeas in many parts of the world, have since been identified.

Of 17 commercially available plant lectins screened by Murdoch *et al* (1990), 5 were found to cause a significant delay in the development of *C.maculatus* larvae at dietary levels of 0.2 % and 1.0 % (w/w). Toxic lectins were specific for GalNAc/gal or GlcNAc. Wheat germ agglutinin (WGA) was the most toxic. Similar levels of toxicity to *C.maculatus* were later observed for rice (*Oryza sativa*) and nettle (*Urtica dioica*) lectin (UDA) which, like WGA, are specific for GlcNAc (Huessing *et al*, 1991). Gatehouse *et al* (1991) have also shown that the winged bean (*Psophocarpus tetragonolobus* DC) lectin which is GlcNAc-specific, to be toxic to *C.maculatus*. The use of lectins specific for N-acetylglucosamine was based on the fact that the insect midgut PM contains chitin, a polymer of GlcNAc (Richards and Richards, 1977) and was thus a target for lectins specific for this amino sugar. However, sugar specificity was shown to be a poor indicator of insecticidal activity. The lectin SNA-II from elderberry, specific for GalNAc, was ineffective against *C.maculatus*, whereas SNA-I, with specificity for 2,60-neuraminyl-gal/GalNAc was highly potent. Similarly the lectin from garden pea has little or no toxic effect, whereas lectins from *Dioclea* spp. are significantly toxic, as is GNA from snowdrop (Gatehouse and Gatehouse, 1998).

A similar approach was adopted by Czapla and Lang (1990) who screened 26 lectins for activity against the southern corn rootworm (SCR) (*Diabrotica undecimpunctata howardii* Barb.). This species is the easiest of the 3 corn rootworm species to rear on artificial diet although the western corn rootworm (*D. vergifera*) and northern (*D. barberi*) cause the greatest economic damage to maize (Czapla, 1997). Of the lectins tested, 3, from castor bean, pokeweed and green marine algae at 2 % in artificial diet were found to be toxic to neonate larvae. An additional 5 lectins, including WGA, inhibited larval growth by at least 40 % compared to larvae fed on control diet. All lectins exhibiting insecticidal

activity were specific for either GalNAc or GlcNAc. The mannose specific snowdrop lectin GNA has also been shown to be insecticidal towards the SCR (Edmonds, 1994). Both GNA and WGA were found by Allsopp and McGhie (1996) to be insecticidal for larvae of the sugarcane whitegrub (*Antitrogus parvulus*) a representative of a complex of 19 melolonthine whitegrub species which are major pests of sugarcane in Eastern Australia. GNA in semi-artificial diet at 0.05 % (v/v) caused significant mortality and growth inhibition after 28 and 22 days, respectively. WGA was active at similar concentrations although expression of the effects was slower Recently an extensive study by Cavaliera *et al* (1995) identified over 60 plant lectins, with different sugar specificities, that inhibited weight gain of neonate SCR by at least 40 % when tested at the 2 % level.

1.7.4 Chitinases as Biopesticides

The activation of plant chitinases in disease resistance has been well documented (Graham and Sticklen, 1994), although their role as an endogenous defence mechanism against insect attack through the use of transgenic plants is only now being realised. A strategy that targets chitin metabolism will give excellent selective pest control due to the limited organs in which the carbohydrate is found. Such examples are the integuments of arthropods and molluscs; the gut linings of insects and the cell walls of fungi and some algae (Kramer and Koga, 1986; Cohen, 1987; Kramer *et al*, 1997).

Whilst the ability of chitinases to degrade gut peritrophic membranes has been redognised for several decades, their potential use as biopesticides has, until recently, remained largely unexplored. In 1993 Shahabuddin demonstrated that the addition of exogenous chitinase (from *Streptomyces griseus*) in a blood meal prevented function of the peritrophic membrane in *Anopheles freeborni in vivo*. Similarly, perforation of the peritrophic membrane was demonstrated when fifth instar *Spodoptera* were feed recombinant endochitinase ChiA*II*.

Chitinases are produced by the insects during molting and during the resynthesis of the peritrophic membrane. The ingestion of excessive amounts of the enzyme has serious physiological consequences for exposed insects, which may in turn lead to death.

The class of chitinolytic enzymes to be used as the biopesticide is important. Kramer (1997) and Powell (1993) demonstrated that bacterial chitinases cause no mortality

when fed to *Oryzaephilus mercator* and *Nilaparvata lugens*. These results may be explained by the abundance of exo-cleaving over endo-cleaving enzymes, the former being substantially less effective than the latter in degrading chitin.

Delivery of the recombinant chitinase is important if this regime is to be used as a pest management strategy. Otsu *et al* (2003) used a chitinase expressing bacteria entrapped in sodium alginate gel beads applied to the leaves of tomato plants to suppress leaf feeding and oviposition by lady beetles. Another mechanism of delivery would be to use plants engineered to express the recombinant chitinase gene. Such transgenic plants bioassays where a recombinant chitinase gene from *Manduca sexta* was expressed in tobacco have returned positive results. Ding *et al* (1997) fed first instar *Heliothis virescens* larvae with leaves of the transgenic plants and after three weeks the total mass of larvae recovered from the chitinase-negative leaves was nearly six fold higher than the mass of the larvae surviving on chitinase-positive leaves. Moreover *O.mercator* larvae feeding upon artificial diet containing an insect derived chitinase will suffer a significant level of mortality when compared to those feeding on a diet containing a plant or microbial chitinase, which have been shown to survive equally as well as those insects feeding upon control diet (Wang *et al*, 1996; Ding *et al*, 1997). The idea of using the insects' own enzyme as a biopesticides is an elegant solution, and one that I feel will prove very effective in the future.

1.8 Research Objectives

The overall aim of this project was to generate an overview of the range of digestive enzymes used by the vine weevil when in its most destructive larval stage. The main objectives of this research are as follows:

To identify the range of hydrolytic enzymes used by the vine weevil during the digestion of plant material.

To study the major proteolytic enzymes found in the vine weevil gut using both biochemical and molecular biology techniques.

To characterise and identify the origin of a cellulolytic enzyme cDNA clone isolated from the vine weevil gut.

Use the knowledge gained from this investigation of the vine weevil gut hydrolyases to generate methods of control through the use of transgenic plants.

2 Materials and Methods

2.1 Chemical Reagents and Equipment Suppliers.

All chemical reagents, except those listed below, were supplied by B.D.H Chemicals Ltd, Poole, Dorset, UK and Sigma Chemicals Co, St Louis, U.S.A. Reagents were of analytical or best available grade.

Antibodies: Anti OC1 – gift from L. Jouanin, INRA, France

Agarose – Gibco BLK Life Technologies Ltd, Paisley, Scotland.

Bacto Agar - -Difco Laboratories, Detroit, Michigan, U.S.A.

Bradford's reagent – Biorad Laboratories Ltd, Biorad House, Mayland Avenue, Hemel Hempstead, Herts, UK.

Developer – Ilford Phenisol – Ilford Lrd, Mobberly, Cheshire, UK.

Disposable pipette tips and Microcentrifuge tubes – Greiner Labortechnik Ltd, Dorsley Gloucestershire, UK.

DNA size markers and restriction enzymes – Northumbria Biochemicals Ltd, Northumberland, UK; Boehringer Mannheim UK Ltd, East Sussex; or Promega.

Fixer, Kodak Unifix – Phase Separation Ltd, Deeside, Clwd, UK.

Lambda ZAP II kit – Stratagene Ltd, 140 Cambridge Innovation Centre, Cambridge Science Park. Milton Rd, Cambridge, UK.

3MM paper and glass wool – Whatmann Ltd, Maidstone, Kent, UK.

Microtitre plates and Petri dishes – Bibby Sterilin Ltd, Stone, Staffordshire, UK.

National Diagnostics "Ecoscint" Scintillation fluid – B.S. & S. (Scotland Ltd), Edinburgh, Scotland.

Nitrocellulose filters, Schleider and Schwell grade BA-85 – Andermann & Co Ltd, Kingston-upon-Thames, Surrey, UK.

Non-fat dried milk powder and nappy liners – Boots Ltd, Nottingham, UK.

PolyATract mRNA isolation kit, Wizard Miniprep DNA purification system, *Taq* polymerase, PCR buffers, dNTP's, MMLV reverse transcriptase – Promega Corporation, 2800 Woods Hollow Rd, Madison, USA.

Radiochemicals and ECL detection kit – Amersham International Plc, Amersham, Bucks, UK.

Sephadex G-50 – Pharmacia Fine Chemicals, Uppsaka, Sweden.

TOPO TA cloning kit – Invitrogen BV, De Schelp 12, Leek, Netherlands.

Trypticase peptone – Becton Dickinson, Cowley, Oxon, UK.

X-ray cassettes – Genetic Research Instrumentations Ltd, Dunmov, Essex, UK.

X-ray films (Fuji-RX) – Fuji Photo Film (UK) Ltd.

Yeast Extract – Umpath Ltd, Basingstoke, UK.

2.2 Treatment of glassware, plastic ware, general apparatus and reagents.

All apparatus coming directly or indirectly in contact with nucleic acids, enzymes, bacteria and sensitive or sterile reagents, were sterilised by autoclaving. Items which could not be autoclaved were sterilised by prolonged immersion or washing in 70% (v/v) EtOH and flamed where possible. All solutions for DNA work, except gels, were autoclaved, or made with sterile water and put through 0.2μm sterilising filter. All water used in making stock solutions was distilled. When RNA was being handled great care was taken to ensure that all apparatus was nuclease free. All glass and plastic ware was soaked O/N in 0.1% (v/v) diethylpyrocarbamate (DEPC) before autoclaving. Similarly all solutions used when handling RNA was DEPC treated and autoclaved.

2.3 Frequently used media, buffers and other solutions.

DNA/RNA electrophoresis

TAE (50x)	2M Tris/acetic acid pH 7.7, 50mM EDTA
DNA gel Loading dye mix	10mM Tris/HCl pH 8.0, 10mM EDTA, 30% (v/v) Glycerol, 0.1% (w/v) bromophenol blue, xylene cyanol FF or fast orange G
Formaldehyde RNA gel loading buffer (5x)	0.5M MOPS pH 7.0, 40mM sodium acetate, 5mM EDTA pH 8.0
Formaldehyde RNA gel running buffer	50% (v/v) glycerol, 1mM EDTA, pH 8.0, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF.

cDNA library screening and southern blotting

SSC (20x)	3M NaCl, 0.3M tri-sodium citate pH 7.0
Denhardts solution (50x)	1% (w/v) each Ficoll 400, polyvinypyrrolidine, BSA
Prehybridisation solution	5x SSC, 5x Denhardts, 0.1% (w/v) SDS, 100μg/ml herring sperm DNA
Hybridisation solution	5x SSC, 1x Denhardts, 0.1% (w/v) SDS, 100μg/ml herring sperm DNA
Denaturation solution	1.5M NaCl, 0.5M NaOH

Neutralising solution	1.5M NaCl, 0.5M Tris/HCl pH 8.0
Wash solutions	2xSSC, 0.1% (w/v) SDS 1xSSC, 0.1% (w/v) SDS 0.1xSSC, 0.1% (w/v) SDS

SDS-PAGE buffers, solutions and size markers

Acrylamides	30g acrylamide, 0.8g bisacrylamide/100ml
Resolving buffer	3M Tris/HCl pH 8.8
Stacking buffer	0.5M Tris/HCl pH 6.8
SDS sample buffer (2x)	0.2M Tris/HCl pH 6.8, 20% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue
Reservoir buffer (10x)	0.25M Tris/HCl, 1.922M glycine, 1% (w/v) SDS
Stain	40% (v/v) MeOH, 7% (v/v) glacial acetic acid, 0.05% (w/v) Kenacid blue
Destain	40% (v/v) MeOH, 7% (v/v) glacial acetic acid
SDS7 protein size markers (Sigma)	66kDa Bovine albumin 45kDa Egg albumin 36kDa Glyceraldehyde-3-phosphate 29kDa Carbonic anhydrase bovine erythrocytes 24kDa PMSF-treated trypsinogen 20kDa Soybean trypsin inhibitor 14kDa Alpha-lactalbumin

Western blotting

Anode buffer 1	0.3M Tris/HCl pH 10.4, 20% (v/v) MeOH
Anode buffer 2	0.025M Tris/HCl pH 10.4, 20% (v/v) MeOH
Cathode buffer	0.025M Tris/HCl pH 9.4, 40mM 6-aminohexanoic acid, 20% (v/v) MeOH

Immunoblot assay

Blocking solution	5% (w/v) Non-fat milk powder, 1xPBS, 1% (v/v) Tween 20
Anti-sera buffer	5% (w/v) Non-fat milk powder, 1xPBS, 0.1% (v/v) Tween 20

Microbial growth media

LB broth	1% (w/v) NaCl, 1% trypticase peptone, 0.5% (w/v) yeast extract, pH 7.5
LB agar	LB broth plus 1.5% (w/v) bacto-agar
Low salt LB broth	0.5% (w/v) NaCl, 1% trypticase peptone, 0.5% (w/v) yeast extract, pH 7.5
Low salt LB agar	Low salt LB broth plus 1.5% (w/v) bacto-agar
NZY broth	0.5% (w/v) NaCl, 10mM $MgSO_4$, 0.5% (w/v) yeast extract, 1% (w/v) NZ amine
NZY agar	NZY broth plus 1.5% (w/v) bacto-agar
NZY top agar	NZY broth plus 0.7% (w/v) agarose
YEB broth	0.5% (w/v) beef extract, 0.5% (w/v) peptone, 0.1% w/v yeast extract, 0.5% w/v sucrose, 2ml/l 1M $MgSO_4$, pH 7.2
YEB agar	YEB broth plus 1.5% (w/v) bacto-agar
YPD broth	1% (w/v) yeast extract, 2% (w/v) peptone, 2% glucose
YPD agar	YPD broth plus 1.5% (w/v) bacto-agar

Antibiotics – Stock Solutions	*Working Solutions*
Kanamycin – 50mg/ml in water	50μg/ml
Carbenicillin – 50mg/ml in water	50μg/ml
Ampicillin – 50mg/ml in water	50μg/ml
Cefotaxime – 500mg/ml in water	500μg/ml
Rifampicin – 100mg/ml in DMF	100μg/ml

Plant tissue culture media

Growth media	1x Murashige and Skoog salts and vitamins, 30g/l sucrose, pH 5.8 with 1M KOH, 8g/l Bactoagar.
Co-cultivation media	1x Murashige and Skoog salts and vitamins, 30g/l sucrose, pH 5.8 with 1M KOH, 8g/l Bactoagar. 1mg/l BAP
Selection media	1x Murashige and Skoog salts and vitamins, 30g/l sucrose, pH 5.8 with 1M KOH, 8g/l Bactoagar. 1mg/l BAP, 200mg/l Cefotaxime, 100mg/l Kanamycin.
Rooting media	1x Murashige and Skoog salts and vitamins, 30g/l sucrose, pH 5.8 with 1M KOH, 8g/l Bactoagar. 0.1mg/l NAA, 200mg/l Cefotaxime, 100mg/l Kanamycin.

Plant growth regulators – Stock Solutions	*Working Solutions*
6-Benzylaminopurine (BAP): 10mg/ml in DMSO	1ml/l
α-Naphthalene acetic acid (NAA): 5mg/ml in DMSO	0.1mg/l

Nickel affinity chromatography buffer set

Binding buffer	20mM Tris-HCl pH 8.0, 0.5M NaCl, 5mM Imidazole, 8M Urea
Wash buffer	20mM Tris-HCl pH 8.0, 0.5M NaCl, 20mM Imidazole, 8M Urea
Elution buffer	20mM Tris-HCl pH 8.0, 0.5M NaCl, 300mM Imidazole, 8M Urea

General buffers

TE buffer	50mM Tris/HCl pH 8.0, 2mM EDTA
Plant protein extraction buffer	20mM Tris/HCl pH 7.5, 1mM DTT

Column buffer	0.15M EDTA, 0.1% SDS, 50mM Tris-HCL, pH 7.5
Phosphate buffered saline (PSB, 10x)	15mM KH_2PO_4, 80mM Na_2HPO_4, 1.37M NaCl
SM buffer	0.58% (w/v) NaCl, 0.2% (w/v) $MgSO_47H_2O$, 5% (v/v) of 1M Tris/HCl pH 7.5, 0.5% (v/v) of 2% (w/v) gelatin

2.4 Molecular Methods

2.4.1 Standard molecular techniques

All basic molecular techniques employed were standard at the Biological Science Department, University of Durham, and were based on protocols in Molecular Cloning: A laboratory Manual (Sambrook *et al* 1989).

2.4.2 RNA extraction

RNA was prepared from 100mg of vine weevil larval guts using Tri-Reagent (Sigma) according to the manufacturers instructions. RNA samples were re-suspended in DEPC treated water and stored in aliquots at -80°C until required.

2.4.3 Isolation of mRNA

Messenger RNA was isolated from total RNA using the PolyATract (Promega) kit according to the manufacturers guidelines. All mRNA samples were stored under liquid nitrogen.

2.4.4 Electrophoresis of RNA

Formaldehyde denaturing agarose gel electrophoresis was used to assess the integrity and concentration of total RNA isolated from the vine weevil prior to mRNA isolation as described by Sambrook *et al* (1989). Electrophoresis was carried out in medium-sized horizontal gel slab apparatus, with a continuous circulation of electrophoresis buffer. HGT agarose gels were prepared at 1.5% (w/v) in 35ml DEPC-treated water and heated in a microwave until melted, once cooled formaldehyde gel loading buffer was added

to a final concentration of 1x, and 10ml of a 37% (v/v) formaldehyde solution was added before pouring into the casting tray. Samples of RNA were prepared by combining with DEPC treated formaldehyde gel running buffer, formaldehyde, and formamide before being loaded onto the gel. Separation was performed at a constant voltage of 3 volts/cm.

After electrophoretic separation the RNA the gel was stained o/n in a 0.5μg/ml EtBr solution, followed by destaining in DEPC treated water. The RNA was then visualised under UV illumination (300nm), and photographed through a red-orange filter (Kodac 23A Wratten).

2.4.5 Electrophoresis of DNA

Electrophoresis of DNA was carried out in horizontal slab gels submerged in buffer as described by Sambrook *et al* (1989). DNA was size fractionated in gels containing 0.7-2.0% (w/v) agarose (Gibco BRL) cast in 1xTAE buffer containing 0.5μg/ml ethidium bromide. Electrophoresis was carried out in a Pharmacia GNA-100 mini-gel or in a NBL medium-sized gel apparatus. A one fifth sample volume of 6x gel loading dye mix was added to the DNA samples prior to loading. The gels were run at a constant voltage (3 volts/cm) in TAE buffer containing 0.5μg/ml ethidium bromide. *Eco*47I digested lambda DNA was generally used as a molecular weight marker. After electrophoretic separation the DNA was visualised under UV illumination (300nm), and photographed through a red-orange filter (Kodac 23A Wratten).

2.4.6 DNA Purification from Agarose Gels

DNA fragments from PCR reactions or restriction digests were isolated from agarose gels using silica fines (0.5-1.0μm particle size). The bands of interest were excised from the agarose gel in a minimal volume and transferred to 1.5ml polypropylene tubes. The DNA fragments were incubated with 1ml of 1M NaI for 10 minutes at 65-70°C. 20μl of the silica fines solution was added and after incubation at room temperature for 20 minutes, with occasional agitation, the bound DNA was pelleted by centrifugation for 1 minute. The pellet was washed in 1ml 70% (v/v) ethanol and air-dried to remove any residue. DNA was eluted by incubating the pellet in 20μl of sterile water for 5 minutes at 37°C. After centrifugation to remove the silica, the supernatant containing the DNA was stored at -20°C.

2.4.7 Quantification of Nucleic Acids

The concentrations of extracted or purified nucleic acids were determined spectrophotometrically by optical density measurement at 260nm using a Beckmann DU 7500 Spectrophotometer assuming an OD_{260nm}=1 (over a path length of 1cm) corresponds to a concentration of 50μg/ml for DNA, 40μg/ml for RNA and 33μg/ml for oligonucleotides. The ratios of A_{260}/A_{280} were used to asses the purity of extracted nucleic acids as determined by Sambrook *et al* (1989).

2.4.8 Plasmid DNA Isolation

Recombinant plasmids and cloning vectors were isolated from their host bacteria using a system based on the alkaline lysis method of Birnboim (1983). Single colonies of bacteria containing the desired plasmid were grown overnight, at 37°C on a rotary shaker, under the appropriate antibiotic selection in 5ml (miniprep) or 50ml (midiprep) of LB broth. Minipreps on 5ml cultures were performed using the Wizard™ DNA purification system (Promega). The QIAfilter Plasmid Midi Kit (Qiagen) was used on 50ml cultures when greater quantities of plasmid DNA were required.

2.4.9 Restriction Enzyme Analysis of DNA

Restriction enzyme digests were carried out using commercially available enzymes and buffers. Typically, digests were performed using 0.5-2.0μg of DNA and 2-10 units of each restriction enzyme. Digests were performed at the recommended temperature for between 2.5-3 hours to ensure complete digestion of the template, before analysis of the fragments by gel electrophoresis.

2.4.10 DNA Sequencing

Sequencing of DNA from plasmid templates was carried out by a modification of the dideoxy chain termination method (Sanger *et al*, 1977) using fluorescent-labelled dye terminators and an AmpliTaq cycle sequencing kit (Perkin-Elmer Applied Biosystems, Warrington, Cheshire, UK). All sequencing was performed using a Perkin-Elmer ABI 373 Stretch or 377 DNA Sequencer by the in-house sequencing service at the University of Durham.

DNA sequencing was used to identify cloned inserts from cDNA libraries, cloned PCR products and to confirm correct incorporation of inserts into expression vectors.

2.4.11 Lambda ZAP cDNA Library Construction

A cDNA library of vine weevil gut tissue was made from approximately 5μg of mRNA using the ZAP-cDNA library construction kit (Stratagene) according to the manufacturers instructions, including all controls except those involved in the packaging of the vector into the phage particles. Once constructed and titred the library was stored in two ways, firstly 2ml aliquots were stored at –80°C with the addition of 7% (v/v) DMSO, the remainder of the library was stored at 4°C in 15ml falcon tubes with 0.3% (v/v) chloroform.

Using this method a library of vine weevil gut tissue was constructed containing 4.5 $x10^6$ (recombinant) pfu/ul after amplification.

2.4.12 Screening of Vine Weevil cDNA library

Recombinant lambda phage were plated out to a density of 50,000 plaque forming units per plate (22x22cm) using host MRF^-, as described by Stratagene. Plaques were grown for 6-8 hours until the lysed zones resembled 'pin-pricks' (approx 0.5-1mm diameter). Growth was then arrested by storing the plates at 4°C overnight. Phage were transferred from the agar plates to nitrocellulose and subsequently denatured and neutralised as described by Sambrook *et al* (1989). During the transfer duplicate lifts were taken to allow accurate identification of positive plaques.

Plaques representing positively hybridised clones were removed from the agar plate and eluted in SM buffer and re-titred to use in a second round of screening. Once plaques had been identified through several rounds of screening the pBlueScript phagmid was excised from the phage genome as described by Stratagene using the *in vivo* excision protocol. Recombinant plasmids were then isolated, and analysed using standard techniques.

2.4.13 Detection of DNA Immobilised on Nitrocellulose Filters

All hybridisation reactions were carried out in Techne hybridisation tubes and ovens (S.H. Scientific, Northumberland), all stages were performed at 65°C and solutions were allowed to reach the working temperature before use. Hybridisation was performed using the procedure described by Sambrook *et al* (1989) using the solutions described earlier. Briefly filters were prehybridised for 2 hours then hybridised with the denatured labelled probe overnight. Subsequently the filters were washed to removed any un-bound or non-specifically bound probe These washes were performed in decreasing salt concentrations as follows; 30 minutes in 2x SSC, 0.1% (w/v) SDS, two times 30 minutes in 1x SSC, 0.1% (w/v) SDS, when Southern Blots were being washed a further wash for 30 minutes in 0.1x SSC, 0.1% (w/v) SDS was included, after which the filters were air-dried.

The dried filters were wrapped in Saran Wrap and fixed to 3MM paper before being secured in film cassettes. To increase the sensitivity of the detection procedure the intensifying screens of the film cassettes were activated by exposure to a low intensity flash light prior to insertion of the X-ray film (Fuji RX). The complete cassette was the stored at -80°C for 5-7 days (cDNA screening) or 3-24 hours (Southern Blots).

Autoradiographs were developed using an automatic developer (X-ograph Imaging systems Compact X4; Malmesbury, Wiltshire, U.K)

2.4.14 Generation of ^{32}P-dCTP labelled DNA probes

Probes for screening cDNA libraries and for analysing Southern Blots were generated from recombinant plasmids containing the verified sequence of interest. The DNA to be used as the probe was digested from the plasmid, and the resulting fragment was isolated after agarose gel electrophoresis. 31μl of the purified DNA (100-250μg) was boiled for 10 minutes and then cooled immediately on ice to leave it in a denatured state. To the DNA 10μl of OLB, 2μl BSA (10mg/ml), 50μCi α^{32}P-dCTP and 2μl Klenow fragment of DNA polymerase (10units/μl) were added and mixed thoroughly The reaction was allowed to proceed overnight at room temperature, prior to termination by the addition of 5μl of 10% (w/v) SDS.

Labelled DNA was separated from unincorporated nucleotides by running the complete reaction mix down a G50 Sephadex column as described by Sambrook *et al* (1989). After separation the specific activity of the probe was calculated by estimation of incorporated counts by scintillation counting of aliquots of the pooled DNA peak. A typical probe had a total count of 1 $x10^8$ cpm.

2.4.15 Standard Polymerase Chain Reaction (PCR)

PCR reactions were typically performed as follows; each reaction (50-100μl) contained PCR buffer consisting of 2.5mM $MgCl_2$, 0.2mM each of dATP, dCTP, dTTP and dGTP, 2 oligonucleotide primers (1μM each), DNA template (50-80ng) and 1 unit of DNA dependent DNA polymerase, where 1 unit catalyses the incorporation of 10μmol of dNTP into acid-insoluble form in 30 minutes at 74°C.

Typically, for amplification from plasmid templates the following PCR conditions were used; 5 minutes at 94°C to initially denature the template, then 30-35 cycles of 94°C for 30 seconds, 55°C for 1 minute for primer annealing, 72°C for 1 minute to allow primer extension, once the cyclic reactions were complete a further extension of 72°C for 9 minutes was used to ensure complete extension of all products and to make sure all products ended with additional adenine residues to facilitate TA cloning. All reactions were carried out using a Perkin Elmer 2400 termal cycler.

For PCR screening and colony PCR *Taq* polymerase was used, when sequences were being amplified for expression constructs Expand polymerase (Roche) was substituted and the extension temperature was dropped to 68°C for targets greater than 2Kbp.

2.4.16 5' Rapid Amplification of cDNA Ends (RACE)

Using 5'RACE the extreme 5' ends of cDNA sequences can be identified. The method was performed as directed by the manufacturer (Clontech), but briefly consisted of the following.

cDNA was generated from isolated target mRNA using a sequence specific anti-sense primer. The SMART oligonucleotide was then annealed to the cytocine residues added to the 5' end of the newly generated cDNA sequence. PCR can then be performed

between the sequence specific primer and a universal primer that is homologous to the SMART oligonucleotide.

2.4.17 Colony PCR

Following subcloning of plasmids into a different host, colony PCR was used as rapid method of identifying bacterial colonies containing correctly recombined plasmids. Between 10-20 transformants were selected and a small biomass from each was resuspended in 25μl sterile water, to which 2x PCR reaction mix was added. PCR was them performed as standard.

2.4.18 Primer Design

Where possible primers were designed to the exact DNA sequence. In the case of primers designed to conserved regions of proteins to allow screening of the vine weevil gut cDNA library and mRNA transcripts for the presence of a particular sequence, the conserved protein sequence was reverse translated into a proposed mRNA sequence allowing for degeneracy in the universal genetic code and where necessary including standard redundant bases.

Primers used in the construction of expression construct were designed with the addition of unique restriction sites, or 6x His tags to aid cloning and purification respectively.

Primer Name	Action of amplification	Sequence (5′ to 3′)
BamHI Oc1	Cloning primer to generate BamHI restriction site at 5′ end of OC1 to allow ligation into pBI121 binary vector.	CCGGATCCATGTCGAGCGACGGAGGGCC
SacI Oc1	Cloning primer to generate SacI restriction site at 3′ end of OC1 to allow ligation into pBI121 binary vector.	CCGAGCTCTTAGGCATTTGCACTGGCATC GACAGGCTTGAACTCCTGAAGCTC

Chitinase A.site	Degenerate anti-sense primer designed to the conserved region of the chitinase active site. To be used in conjunction with 5'RACE universal primer (Clontech)	GGRTAYTCCCARTCXAXRTCXAXXCCRTCR AA
Cathepsin race rev	Degenerate anti-sense primer towards the 5' end of a typical cathepsin sequence. Used to determine terminal 5' sequence using 5'RACE	GGRTAYTCCCARTCXAXRTCXAXXCCRTCR AA
5'ChiExp	Cloning primer to generate BamHI restriction site at 5' end of vine weevil chitinase clone to allow ligation into pBI121 binary vector.	CCGGATCCATGGGCAAAAGGTTAATTC
3'ChiExp	Cloning primer to generate SacI restriction site at 3' end of the vine weevil chitinase clone to allow ligation into pBI121 binary vector.	CGAGCTCTTAGCAAACAACTGCGGAGATC CAGTTGCA
Pit 5'Cell	Cloning primer to fuse the vine weevil cellobiohydrolase clone to the α-factor leader sequence of pGAPzαA	CTCGAGAAAAGAGAGGCTCAAGCTGGAA CATACCTAGATCGC
Pit 3'Cell	Primer for the 3' end of the vine weevil cellobiohydrolase sequence for cloning into pGAPzαA, including a 6xHis tag prior to the stop codon	TCTAGACTAGTGGTGGTGGTGGTGGTGTT GTTCGAACAGGATACCG

Cellulase5′ NdeI	Cloning primer to introduce the vine weevil cellobiohydrolase cDNA into the bacterial expression vector pET24a. Primer includes a novel NdeI site at he terminal 5′ end	CATATGGGAACATACCTAGATCGCTTCAA GG
Cellulase3′ 6xHis	Primer for the 3′ end of the vine weevil cellobiohydrolase sequence for cloning into pET24a, including a 6xHis tag prior to the stop codon	CTCGAGTCAGTGGTGGTGGTGGTGGTGTT GTTCGAACAGGATACCGTAGG
Cellulase Int For	PCR/Sequencing primer to identify the previously unsequenced internal region of clone VW24	CGTCGGCGACAACAGCTACACC
Cellulase Int Rev	PCR/Sequencing primer to identify the previously unsequenced internal region of clone VW24	GCAACCCATTTCTTCAAGATGG

2.4.19 Ligation of DNA

Ligation of DNA fragments were usually performed in 10μl reactions, using commercially available T4 DNA ligase in appropriate buffers. Digested, purified vector and insert(s) were added at a molar ration of 1:3, to 1 unit of T4 DNA ligase and 1μl of 10x ligase buffer, after adjusting the final volume to 10μl with sterile water the reaction was allowed to proceed overnight at room temperature.

2.4.20 TOPO-TA cloning

Typically, fresh PCR and RT-PCR products were cloned into the TA cloning vector pCR2.1 (Invitrogen) which has topoisomerases linked to overhanging T residues to accept the PCR product with it's extended A overhangs which are added by the *Taq*

polymerase. This kit allows ligation of the target sequence into the vector in around 5 minutes, and when used in combination with TOP10 high efficiency competent cells gave a reliable method for cloning and propagating important PCR amplification products.

2.4.21 Transformation of E.coli

Transformation of DNA into chemically competent *E.coli* was performed using standard techniques as described by Sambrook (1989). Where possible blue/white selection was used to identify recombinant bacterial colonies, for this 40μg/ml 5-bromo-4-chloro-3-indolylß-D-galactoside (X-Gal) and 0.1mM isopropyl-ß-D-thiogalactoside (IPTG) were added to the agar plates containing the appropriate antibiotic.

2.4.22 Transformation of Agrobacteria

Electrocompetent cells of *Agrobacterium tumefaciens* (strain LBA4404) were generated following the method of Wen-jun and Forde (1989). Briefly bacteria were grown overnight to an OD_{600} of 1.0 under rifampicin selection. The cells were then spun down and resuspended in decreasing volumes of ice cold 10% (v/v) glycerol, until the total volume had been reduced to 0.01 times that of the original. Electrocompetent cells were stored at –80°C until required.

Transformation was performed using a Bio-Rad Gene Pulsar under the following conditions; a field strength of 12.5kV/cm, a capacitance of 25μF and resistors of 400 or 600 ohms in parallel with the sample. 1-5μl of supercoiled plasmid was used per transformation event, and after incubation in SOC media for 3 hours the recombinant bacteria was selected for using the appropriate antibiotic.

2.4.23 Genomic DNA Extraction

Genomic DNA was extracted from adult vine weevils using a plant genomic DNA extraction kit (Sigma) with an additional chloroform extraction to remove fatty residue. DNA was extracted separately from the guts, heads and bodies of adult vine weevils. The DNA isolated was of a purity suitable for Southern blotting and the construction of a genomic lambda phage library.

2.4.24 Southern Blotting

1μg of vine weevil genomic DNA isolated from adult heads and adult bodies was digested for 3 hours with the following enzymes; *HincII, HindIII, HindIII* and *EcoRI, BamHI*. The resulting products were run on a 0.8% (w/v) agarose gel with control *HincII* digested VW24 cDNA clone, visualised and photographed as standard. The DNA was transferred to a nitrocellulose membrane as described by Sambrook *et al* (1989).

The membrane was subsequently probed using a ^{32}P-dCTP labelled *HincII* fragment of the VW24 cDNA. After washing to a high stringency the membrane was exposed to x-ray film for 4 - 96 hours at -80°C in an image intensifying film cassette, which was processed in the usual manner.

2.4.25 Vine Weevil Genomic Library

A phage library of vine weevil genomic DNA was constructed using the ZAP Express Pre-digested Vector kit (Stratagene #239212), strictly following the manufacturers experimental procedure. Briefly, DNA from adult vine weevil guts and DNA from adult vine weevil heads and bodies was digested with *BamHI*, to give compatible ends for ligation to lambda arms, and phenol/chloroform extracted to remove the restriction enzyme. 150ng of restricted gDNA from each source was ligated into the pre-digested ZAP Express vector, which was then packaged into Gigapack III Gold Packaging Extract.

The library was then handled, stored and screened as with the cDNA library using the methods described by the manufacturer and those of Sambrook (1989).

2.5 Protein Analysis

2.5.1 SDS-PAGE Analysis

Proteins were analysed by sodium dodecyl sulphate polyacrylamide gel electrphoresis (SDS-PAGE), run in a dissociating SDS-PAGE discontinuous buffer system (Laemmli, 1970). Minigels (9x10 cm) were as described by Hames (1981) and run in an ATTO AE-6450 vertical gel tank apparatus (Genetic Research Instrumentation Ltd, Essex). Protein

samples were prepared by adding an equal volume of 2x SDS sample buffer, 10% (v/v) ß-mecaptoethanol was added when the samples were in need of reducing. Samples were then boiled for 10 minutes before being loaded onto the gel. Gels were typically run at 70 volts until the protein samples had migrated through the stacking gel, and then the voltage was increased to 100-120 volts until electrophoresis was complete.

2.5.2 In Gel Enzyme Assays

Cellulolytic activity in crude gut extracts and in purified recombinant proteins was detected directly by a modification of the method described by Girard (1999). SDS-polyacrylamide gels (12.5% gels) were prepared and run by standard protocols (Laemmli 1970) except that 0.1% (w/v) carboxymethyl cellulose was dissolved in the gel mix prior to polymerisation. After running, gels were soaked in a 2.5% (v/v) solution of Triton X-100 for 30 minutes to remove SDS from the gel. Gels were then soaked in activation buffer (100mM sodium-phosphate pH 6.0) for 2 hours at 37°C before staining in 1% (w/v) congo red. Finally gels were destained in 1M NaCl to reveal area of lysed substrate, before photographing.

2.5.3 Protein Detection in Polyacrylamide gels

Protein bands were visualised by staining with 0.05% (w/v) Kenacid blue, in 7% (v/v) acetic acid, 40% (v/v) MeOH followed by de-staining in 7% (v/v) acetic acid, 40% (v/v) MeOH (Sambrook 1989). Once de-stained gels were scanned using an Agfa flat-bed scanner.

2.5.4 Western Blotting

Electrophoretic transfer of proteins from a protein gel to a nitrocellulose membrane (grade BA85, Schleicher and Schuell Inc.) was performed using the semi-dry blotting technique of Kyhse-Anderson (1974), in an ATTO blotting apparatus. The transfer stack was constructed in the following order; Anode plate, 2 sheets of 3MM paper soaked in anode buffer 1, 1 sheet of 3MM paper soaked in anode buffer 2, 1 sheet of nitrocellulose soaked in distilled water, the gel, 3 sheets of 3MM soaked in cathode buffer, Cathode plate. Proteins were electro-transferred at 125-150 mA for 1 hour, after which the gel was stained to check for successful protein transfer and the nitrocellulose membrane was stored between 3MM paper at 4°C.

2.5.5 Immunodetection of Nitrocellulose Bound Proteins

Nitrocellulose membranes were incubated for 1 hour at room temperature in an excess volume of blocking solution, after which the primary antibody was added at the appropriate dilution in anti-sera buffer. The membrane was incubated in the primary anitibody solution at room temperature for 1.5 hours with gentle rocking. Unbound antibody was then removed by washing the membrane in anti-sera buffer for 3x 5 minutes at room temperature. Secondary IgG horseradish peroxidase conjugate (BioRad) was used to detect bound primary antibody, and was incubated with the membrane for 1.5 hours followed by three washes in 1xPBS, 0.1% Tween 20, and a final rinse in distilled water. Enhanced chemiluminescence (ECL) reagents (Amersham) were used to detect the bound antibody complexes, and bands were visualised by exposure to X-ray film (Fuji) which were developed as described earlier.

2.5.6 Protein Concentration Determination

Protein concentration in solutions were determined using a commercial dye-binding assay (BioRad) based on the Bradford (1976) assay. Bovine serum albumin (BSA) was used as a protein standard, from which a calibration curve was constructed between $0\mu g/ml$ and $2.5\mu g/ml$. Dilutions of BSA were prepared in the same buffer as that used for the sample preparation. In duplicate individual wells of a microtitre plate, protein samples were diluted to a volume of $160\mu l$ in distilled water, to this $40\mu l$ of Bradford reagent were added. The plate was shaken briefly to ensure thorough mixing of all reagents, and the samples measured at 570nm using a Dynatech MT 5000 microtitre plate reader. Empty wells were used as blanks.

2.6 Heterologous Expression of VW24

2.6.1 Expression of Gene Products in E.coli

The coding sequence of VW24 (Cellobiohydrolase) was amplified from the pBlueScript vector using primers specific to the 5′ and 3′ regions. The primers were designed to include unique restriction sites to allow directed cloning, the 3′ primer was also engineered to contain a 6x His tag after the end of the VW24 coding sequence and before the consensus stop codon. The resulting product was ligated into the pET24d

(Novagen) bacterial expression vector, which was subsequently transformed into BL21(DE3) to allow heterologous expression of the VW24 gene product.

A single recombinant colony was used to inoculate 10ml LB medium which was grown overnight at 37°C, 5ml of this culture was then used to inoculate 500ml LB medium with the appropriate antibiotic selection in a baffled shake flask. The recombinant bacteria was grown at 37°C until midlog phase (OD_{600}=0.6), when IPTG was added to a final concentration of 1mM to induce production of the recombinant protein. Induction was performed for a further 3 hours at 37°C.

After induction bacterial cells were collected by centrifugation and resuspended in nickel affinity column binding buffer. Bacterial cells were disrupted by sonication and insoluble material was removed by centrifugation after which the resulting precipitatioe was dissolved in binding buffer plus 8M urea.

2.6.2 Expression of Gene Products in Pichia pastoris

The gene product of VW24 was expressed in *Pichia* in a similar fashion to bacterial expression, however the VW24 ORF was fused to the α-factor protein targeting sequence of pGAPZαA (Invitrogen). Baffled shake flasks 250ml YPD medium were inoculated with single colonies of recombinant *Pichia*, and grown at 30°C for 5 days with continual shaking (300rpm). Cells were then removed from the supernatant, which contained the recombinant proteins. Typically the proteins within the supernatant were precipitated by the addition ammonium sulphate to 90% saturation.

2.7 Purification of Recombinant VW24 Gene Product

2.7.1 Nickel affinity column chromatography

Recombinant proteins produced by bacterial expression were purified by nickel column chromatography under denaturing conditions. Briefly, solublilized protein extracts were applied to a FPLC column containing immobilised $Ni^{2=}$ (vol. 5 ml). Once loaded the column was washed with binding buffer (5mM imidazole) until all unbound protein was removed. The column was then washed in washing buffer (20mM imidazole)to remove any non-specially bound protein, and after this the bound

recombinant protein was eluted in 300mM imidazole and collected in fractions. Elution peak fractions were routinely analysed on SDS-PAGE gels.

2.7.2 Reverse phase column chromatography

The protein content to the *Pichia* supernatant was enriched by ammonium sulphate precipitation (60g/100ml) overnight at 4°C, the precipitated proteins was then collected by centrifugation and resuspended in 4M NaCl to be loaded on to a phenyl sepharose column, using hydrophobic interaction chromatography. Recombinant protein solution was loaded and washed in 4M NaCl until all non-bound material had eluted. The column was then subjected to linear decreasing concentrations of salt to remove bound proteins. The eluted proteins were collected in 1ml fractions which were analysed to for their ability to hydrolyse the 4-methylumbelliferyl cellobioside substrate.

2.8 In Vitro *Vine Weevil Gut Enzyme Assays*

2.8.1 Vine Weevil Gut Protease Assay

Protease assays were performed *in vitro* using the Enzchek Protease Assay kit (casein-BODIPY Green Fluorescence, Molecular Probes). The fluorescent dye used exhibits strong, pH independent fluorescence (Jones *et al* 1997) in its free form with excitation and emission spectra of 505nm and 513nm respectively. Protease activity was measured using a microtitre plate reader (Menges *et al* 1997) with enzyme activity proportional to rate of fluorescence increase.

Assays were initially performed over a wide pH range to identify conditions in which *in vivo* proteolysis may occur. The following buffer systems were employed; 0.1M acetate pH 3.5/4.0/4.5/5.0/5.5, 0.1M Bis-Tris propane buffer pH 6.0/6.5/7.0/7.5/8.0/8.5/9.0/9.5 and 0.1M CAPS buffer pH 10.0/10.5. Each buffer was supplemented with 1mM cysteine, 1mM EDTA, and 0.05% (v/v) Brij 35. Assays were performed in a total of 200μl and typically consisted of 180μl reaction buffer, 10μl casein-BODIPY substrate and 10μl of diluted crude gut extract (5 vine weevil larval gut homogenised in 100μl water and chloroform extracted, then diluted 30 x in water). Fluorescence was read every minute for 30 minutes using excitation and emission filters of 485nm and 538nm respectively.

The assay was repeated under conditions that gave highest levels of fluorescence, in the presence of specific protease inhibitors. The chemical inhibitor E64 was used to inhibit cysteine proteases, and the plant derived serine protease inhibitor SKTI (Soybean Kunitz Trypsin Inhibitor) was used to inhibit the activity of trypsin proteases. Inhibition assays were performed at pH 4.5 and pH 9.5 as described earlier with the addition of 10^{-6}M E64 or 10^{-6}M SKTi, or both inhibitors together in the same assay. Assays were typically performed in duplicate and the fluorescence was measured at 2 minute intervals for 60 minutes.

2.9 Vine Weevil Gut Cellulase Activity

2.9.1 Cellobiohydrolase and ß-glucosidase activity

The presence of cellobiohydrolase (CBH) cellulolytic enzymes in the vine weevil gut was analysed using the synthetic substrate 4-methylumbelliferyl-ß-D-cellobioside (Sigma), and ß-glucosidase activity was analysed using 4-methylumbelliferyl-ß-D-glucoside (MuGlu, Molecular Probes). Similarly to the proteolytic activity of the gut cellulolytic activity was measured over a wide pH range, and assays were performed as follows, 1μl of undiluted crude gut extract was combined with 1mM substrate in a total of volume of 200μl. Assays were typically performed in duplicate with the appropriate controls. The fluorescence of 4-methylumbelliferone is pH dependent, with maximal fluorescence seen in strong alkaline conditions. To overcome this phenomenon assays were incubated 30°C for 40 minutes before each was stopped with 100μl 0.1M NaOH to increase the actual fluorescence and read once using the excitation and emission filters of 355nm and 460nm respectively, or assays were kinetically read over a set time frame at the sub-optimal fluorescence.

2.9.2 Total cellulose digestion

The ability of total cellulase digestion to occur *in vivo* in the vine weevil gut was analysed using the Amplex Red Total Cellulose Digestion Kit (Molecular Probes).

2.10 Tobacco Leaf Disk Transformation

Leaf disc transformation was performed on tobacco plants (*N.silvestra*) as described by Gallois and Marinho (1995), a simplification of Horsch *et al* (1985). Briefly it consists of

immersing the leaf disks in a liquid culture of *Agrobacterium* (strain LBA4404) carrying the chosen transformation vector. The plant tissue and *Agrobacterium* were then cocultivated on regeneration medium for a period of 2 days at the end of which leaf disks are transferred to regeneration medium, supplemented with an antibiotic to kill the bacteria (Cefotaxime), and the plant specific vector born selective agent. Using this method rooted plantlets may be obtained within 2 months.

3 Identification of Digestive Enzymes in Vine Weevil by a Random Selection cDNA Approach

To investigate the array of enzymes utilised by the vine weevil during the digestive process a non-specific screening protocol was required. This was achieved by analysing the sequences of clones isolated at random from the cDNA library prepared using mRNA isolated from larval guts. This approach would give a "snap shot" of the mRNA population in the tissue from which the library was constructed. Although such a survey is incomplete, common mRNA species should be represented. With the library being generated from gut tissue, a relatively large proportion of the clones analysed may encode the digestive proteins secreted into the gut lumen, which will represent a major part of protein synthesis.

3.1 Random screening of the gut cDNA library

A sample of the cDNA library equivalent to $1x10^7$ pfu was excised into the pBluescript SK plasmid, as described by the manufacturers guidelines (Stratagene, Manual # 200401). Briefly ExAssist helper phage is used to excise the cloned cDNA and its flanking regions from the phage genome, which is subsequently circularised to generate the pBluescript SK plasmid. The cloned cDNA inserts can then be analysed through restriction analysis and sequencing.

The mass excision of the vine weevil cDNA library produced several hundred recombinant colonies when grown on selective media (LB ampicillin 75μg/ml) with blue/white selection. Twenty five of these clones were grown overnight in 10ml of LB broth to allow extraction of the plasmids using the Wizard miniprep kit. Plasmid DNA was digested with *PstI* and *XhoI* to release the cloned cDNA insert, resulting fragments were visualised by electrophoresis on a 1% EtBr agarose gel. Figure 3-1 shows the resulting banding pattern obtained from the restriction digests. Those plasmids containing inserts larger than 700bp were chosen for partial sequencing from the 5′ end.

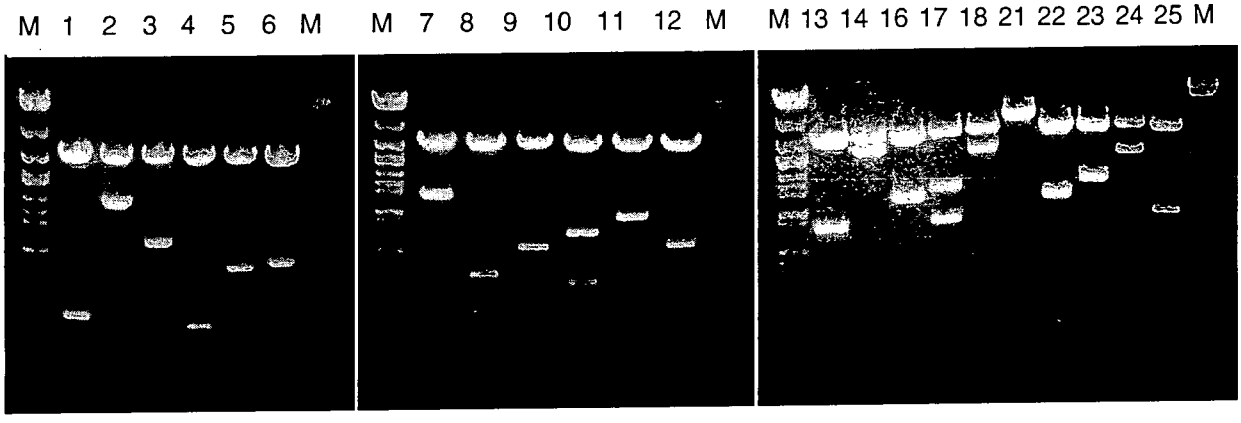


Figure 3-1 Agarose gel (1%) showing the restriction fragment profile of the excised clones from the random screening of the vine weevil cDNA library, after digestion with PstI and XhoI. Lanes M contain the molecular weight marker Eco471, lanes; 1 - 14, 16 – 18, 21 – 24 and 25 contain the digested plasmids. Clones are named by the corresponding lane with the prefix 'VW' e.g. lane 1 contains clone VW1.

3.2 Analysis of excised clones

Of the twenty two clones restricted with *PstI/XhoI* all but one, VW21, released non-vector insert fragments. These fragments were between 700bp and 2.7Kbp, in some instances two fragments were seen after digestion and were presumed to be a product of digestion at an additional internal *PstI/XhoI* site. Table 3.1 shows the approximate molecular sizes of each of the cDNA inserts, where two fragments were present the molecular size represents the sum of both products.

All clones, except VW21 which contained no distinguishable insert, and VW1 and VW4 whose inserts were deemed too small to encode peptides of significant importance were partially sequenced from their 5' ends using the SK sequencing primer. Comparison of the derived sequences showed that each of the nineteen clones contained an insert derived from a different mRNA species. Subsequent comparison of the protein sequences encoded by the clones to the non-redundent protein sequence database (www.ncbi.nlm.nih.gov) using BlastX highlighted nine clones that could be putatively identified as proteins with a known function (Table 3.2). Six of these clones showed similarity to proteins with a putative role in digestion; two serine proteases, a cellobiohydrolase, a pectinesterase, a lipase and an acid phosphatase (see annex 8.1.3), while the other three clones showed similarity to more diverse enzymes. Two of these clones encoded proteins which formed part of the protein synthesis system (ribosomal protein (see annex 8.1.1), elongation factor 2 (see annex 8.1.4)): the final clone encoded cytochrome C oxidase subunit II (see annex 8.1.2). From the remaining ten clones, four showed either no significant similarity to known proteins or showed similarity to proteins which had not been functionally characterised and were not studied further (Table 3.2). The final six clones showed unusually high levels of A/T rich regions (64 to 79% of the total sequence), and/or had no significant open reading frames, therefore were unlikely to encode proteins. No similarity was found to these clones in the databases (Table 3.2).

Clone Number	Number of Non-Vector Gel Bands (Fig 1)	Total Estimated cDNA Insert Length (base pairs)
VW1	1	400
VW2	1	1600
VW3	1	1100
VW4	2	700
VW5	1	700
VW6	1	800
VW7	1	1600
VW8	2	600
VW9	2	800
VW10	2	1000
VW11	1	800
VW12	1	600
VW13	1	800
VW14	1	2600
VW16	2	2700
VW17	2	2200
VW18	2	3100
VW21	0	-
VW22	1	1100
VW23	1	1400
VW24	2	2000
VW25	2	1700

Table 3.1 Summary of the restriction fragment length profile as seen after agarose gel electrophoresis following digestion of excised phagmid clones with PstI and XhoI. All sizes are approximate and displayed to the nearest 100 bases.

Clone Identity	Putative Homology	Putative *in vivo* Function	% Identity in 5 Highest scoring regions (amino acids)	Mean length of similarity region (amino acids)
VW24	Cellulase	Cellulose degradation	55-65	231
VW5	Trypsin	Protein degradation	39-52	237
VW11	Trypsin	Protein degradation	34-37	210
VW16	Pectinesterase	Pectin degradation	35-44	156
VW10	Lipase	Lipid degradation	43-52	211
VW8	40S Ribosomal Protein (S15)	Compenent of ribosome, protein synthesis	66-74	127
VW9	Cytochrome C oxidase subunit III	Respiratory chain	41-42	236
VW18	Acid Phosphatase	Phosphoester cleavage in lysosome	35-37	118
VW14	Elongation Factor 2	Protein elongation factor	73-78	239
VW2, VW6, VW13, VW25	None/Uncharacterised	Uncertain		
VW3, VW7, VW12, VW17, VW22, VW23	Low complexity/Non-coding	None		

Table 3.2 Summary of the putative functions of clones isolated from the *O.sulcatus* larval gut cDNA library, by similarity of partial 5′ translated sequence to known proteins after searching the online Blastx database.

3.3 The serine proteases, VW5 and VW11

The polypeptides encoded by these 5′ partial sequences showed similarity to serine proteases. These sequences are described in detail in Chapter 4

3.4 The pectinesterase (or pectinmethylesterase), VW16

The polypeptide encoded by the 5′ partial sequence of VW16 showed strong similarity to several pectinesterase enzymes from bacteria and plants. Pectinesterases participate in the breakdown of pectin, a major component of the middle lamella of plant cell walls. The most similar sequences (highest scores by BlastX comparisons) were all proteins either characterised as pectinesterases (*E.crysanthemi*) or putative pectinesterases (*Salmonella sp.*), and showed similarities towards more than 35% of the vine weevil sequence. These factors suggest homology and allow VW16 to be putatively identified.

VW16 was subsequently sequenced from the 3′end to allow a contiguous sequence of approximately 1.2kb to be assembled, based upon a 114bp overlap between the two partial sequences. The insert size is consistent with that predicted from the restriction digest analysis of the recombinant plasmid (Figure 3-1), if a minor band of 1.5Kbp is ignored. With no additional *PstI* or *XhoI* restriction sites identified in the actual sequence, it was concluded that the minor band of 1.5Kb (Figure 3-1) is likely to be an artefact of incomplete digestion. Sequence analysis of the complete insert showed a large incomplete ORF (lacking an obvious translation start codon) extending from the 5′end of the sequence to nucleotide position 1085 with a putative polyA tail of 22 consecutive adenine residues starting 51bp downstream of the primary stop codon. Two consensus polyadenylation signals (AATAAA) were identified in the terminal 3′ region of the sequence at 17 and 50bp upstream of the polyA tail.

The cDNA transcript encoded a truncated preprotein of 361 amino acids in length with a molecular weight of 39KDa and an isoelectric point (pI) of 6.87. Analysis using the SignalP software indicated no distinguishable signal peptide, but the polypeptide is truncated at the N-terminus, and it is predicted that the full sequence clone contains a signal peptide.

When the non-redundant protein database was searched with the complete predicted polypeptide sequence, the initial identification of the VW16 sequence as encoding a

pectinmethylesterase was confirmed, with the same database sequences identified as most similar.

Figure 3-2 shows multiple alignments between the translated VW16 clone and selected bacterial pectin methylesterases (based upon Blast results). It highlights several positions where certain residues are conserved between all or most of the different enzymes (homology based). Of these residues a particularly well conserved region is seen between residues 225 and 240 of VW16 where sequence similarity is greater than 65% from all species (Figure 3-2). Further analysis of this region through PROSITE (www.expasy.ch/prosite) shows that residues 225-234 match a signature region of [IV]-x-G-[STAD]-[LIVT]-D-[FYI]-[IV]-[FSN]-G (where bracketed residues are alternatives) found within all known members of the pectin methylesterase family (prosite PS00503). A second region characteristic of pectinesterases, PS00800 [GSTNP]-X(6)-[FYVHR]-[IVN]-[KEP]-X-G-[STIVKRQ]-Y-[DNQKRMV]-[EP]-X(3)-[LIMVA] is also present in VW16 at residues 77-96.

Figure 3-2 Multiple alignments between the predicted protein product of VW16 with selected bacterial pectinesterases. Sequences were aligned using the Clustal V (MegAlign, DNA Star.), dark highlights indicate residues fully conserved in all aligned sequences. The solid line indicates Prosite pattern matched by all aligned sequences. Sequences are as follows: P07863, Pectinesterase A precursor of *E.crysanthemi*, Q47474; Pectinesterase B precursor of *E.crysanthemi*, P24791; Pectinesterase precursor of *Ralstonia solanocaenum*.

3.5 The lipase, VW10

The polypeptide encoded by the 5′ partial sequence of VW10 was similar to a number of triacylglycerol lipases of mammalian and insect origin (Table 3.2). Of these similar sequences, the highest Blast score was seen with Lipase 3 from *Drosophila melanogaster* (52% identity in a 20 amino acid overlap with an E value of $9x10^{-55}$). This high degree of similarity suggests that VW10 may be the vine weevil homologue of the *Drosophila* gene.

VW10 was also sequenced from the 3′ terminus, and the resulting sequence was aligned to the 5′ partial sequence producing a contig of approximately 1.2kb, based on an overlap of 205bp. An additional internal *PstI* restriction site at position 792 is consistent with the evidence gained from the initial restriction digest (Figure 3-1) where two products of approximately 800bp and 400bp were seen. The contig contained a large incomplete ORF, lacking an obvious start codon, followed by a putative 3′UTR of 21bp and a polyA tail of 17 consecutive adenine residues. Although the 3′UTR exists, it is very short and does not contain a consensus poly-adenylation signal.

Once translated, the ORF represented a predicted truncated protein of 375 amino acids with a molecular weight of around 42KDa and a pI of 6.1. Comparison of the sequence encoded by VW10 with entries in the non-redundant protein database showed that homology extended over almost the entire length of several lipases. Of these sequences highest similarity was seen to the lysosomal acid lipase 3 of *D.melanogaster* (44% identity over 364 residues). Similarity to a number of secreted digestive lipases was also seen, the highest level of similarity being to lipase 1 of *D.melanogaster* (41% identity over 373 residues). The homology to lipases is shown in Figure 3-3 and Figure 3-4 where the translation product of VW10 has been aligned with four lysosomal lipases (Figure 3-3) and also with four secreted digestive lipases (Figure 3-4). The two separate alignments indicate that VW10 lacks an N-terminal signal peptide sequence as seen in all other lipases analysed here (Figure 3-3 and Figure 3-4); this is concurrent with the fact that no signal peptide was identified using SignalP software. The absence of a start codon confirms that VW10 is truncated at the N-ternimus.

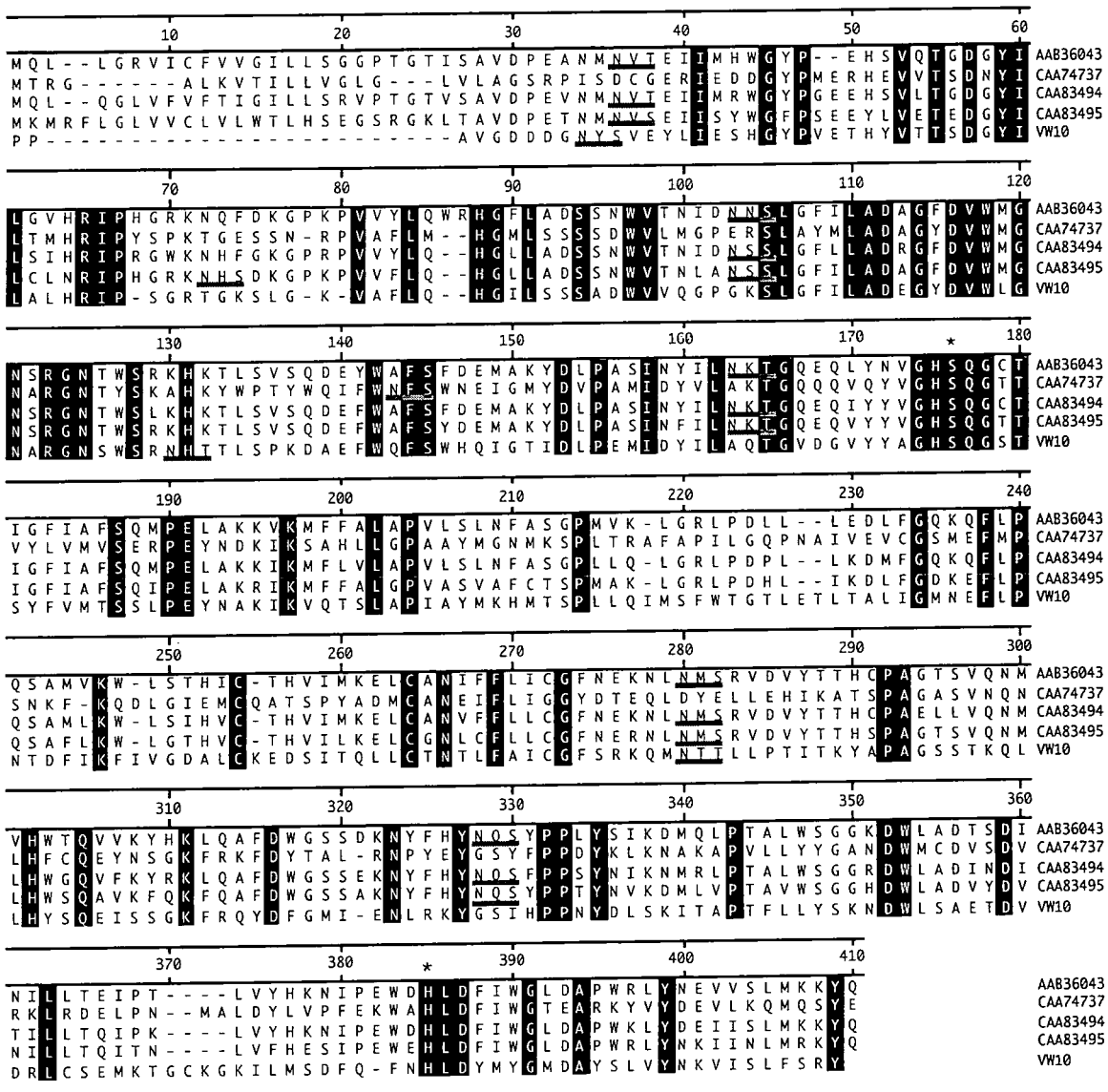


Figure 3-3 Multiple alignments of VW10 predicted protein product with four lysosomal acid lipases of insect and mammalian origin. AAB36043; lysosomal acid lipase, *Rattus sp.*, CAA74737; lipase 3, *Drosophila melanogaster*, CAA83494; lysosomal acid lipase, *Mus musculus*, CAA83495; lysosomal acid lipase, *Homo sapiens*. Alignment was performed using ClustalV, dark shaded areas indicate fully conserved residues. Catalytic residues are marked with asterisks (*) as identified through similarity, and putative *N*-glycosylation sites are highlighted.

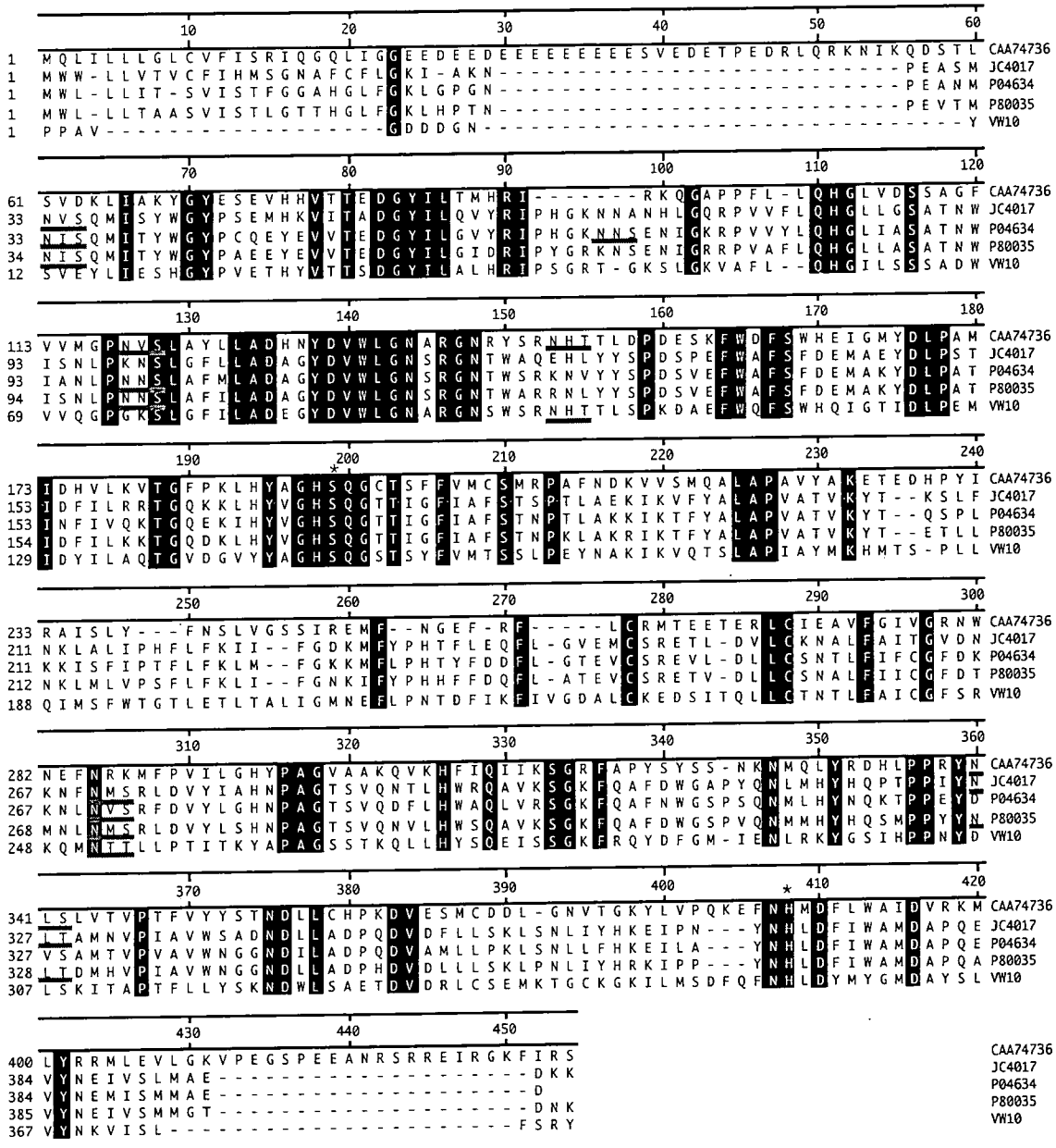


Figure 3-4 Multiple alignments of VW10 predicted protein product with four similar digestive lipases of insect and mammalian origin. CAA74736; lipase 1, *Drosophila melanogaster*, JC4017; triacylglycerol lipase, *Bos taurus*, P04634; triacylglycerol lipase, lingual precursor (Lingual lipase), *Rattus norvegicus*, P80035; triacylglycerol lipase, gastric precursor (Gastric lipase), *Canis familiaris*. Alignments were performed using ClustalV, dark shaded areas indicate fully conserved residues. Catalytic residues are marked with asterisks (*) as identified through similarity, and putative *N*-glycosylation sites are identified.

VW10, and the secreted digestive lipase from *C.familiaris*, *R.norvegicus* and *B.Taurus* do not align with a glutamate (Glu, E) rich region of 26 residues found at the N-terminus of lipase 1 from *D.melanogaster*. However, this glutamate rich region is thought to be unique to the *Drosophila* product (Pistillo *et al* 1998). Figures 3.3 and 3.4 show several regions of absolute homology and conservation between species particularly the area bounded by residues 42 and 46 of VW10 where the highly conserved region of GHSQG is located. Within this region the serine residue has catalytic function (by similarity), and forms a catalytic triad with a histidine (His, H) and an aspartate (Asn, D) in the active site of the enzyme (Frenken *et al* 1992). The histidine residue of the catalytic triad in VW10 was putatively identified as H_{351} through pair-wise alignment with the lipase from *Chromobacterium viscosum* (data not shown) for which the catalytic residues have been identified (Lang *et al* 1996). Figures 3.3 and 3.4 show that VW10 His_{351} is well conserved amongst the multiple alignments shown. Pair-wise alignment with *C.viscosum* lipase did not however indicate a candidate for the active aspartate residue in VW10, though multiple alignments between known lipases show that all sequences chosen contain several highly conserved aspartate residues. These conserved aspartate residues may be substituted for the previously identified catalytic residue. Also Noble (1996) demonstrated that the aspartate residue of the catalytic triad is not necessary for lipase activity.

3.6 The cellulase, VW24

The polypeptide encoded by the 5′ partial sequence of VW24 showed strong similarity to eleven putative or fully characterised cellulase enzymes of bacterial origin. All the sequences showing significant similarity were members of the gylcosyl hydrolase family 48 which are grouped by sequence similarity (Henrissat, 1991). This family of glycosyl hydrolases contains cellobiohydrolases, and endoglucanase type enzymes, which act synergistically to degrade insoluble crystalline cellulose into small soluble glucan units.

VW24 was sequenced in the reverse direction giving some 700 reliable bases from the 3′end of the cDNA, however no overlap was seen between it, and the 700bp of the initial forward sequencing run from the 5′end. The restriction analysis of VW24 (Figure 3-1) suggests that the total length of the clone is 2kb, thus indicating that around 600bp of additional internal sequence would be needed to construct a complete contiguous sequence. A pair of internal sequencing primers were designed to the 3′ region of the

forward sequence (5′ CGTCGGCGACAACAGCTACACC 3′) and the 5′ region of the reverse sequence (5′ GCAACCCATTTCTTCAAGATGG 3′). PCR using the two primers amplified the expected 600bp product (data not shown), the primers were subsequently used independently to sequence the internal region of VW24 in both directions.

Once assembled with the partial 5′ and 3′ sequences the internal sequencing of VW24 revealed a contiguous sequence of 1,987bp, as predicted by the restriction analysis. The contiguous sequence contains a possible incomplete ORF of 1911bp from the terminal 5′ end to a TAA stop codon, however this ORF lacks an obvious start codon. The sequence contains a polyA-tail of 24 consecutive adenine residues starting at position 1964 bases. 20bp upstream of this position, located in the 3′UTR of 50 bases, is a single consensus polyadenylation signal (AATAAA bases 1945-1951).

Translation of the ORF wa predicted to give a putative truncated product of 638 amino acids in length, with a predicted molecular weight of around 71KDa and a pI of 4.6. SignalP analysis predicts an ER entry signal of at least 17-18 residues (Figure 3-5) with the cleavage of the peptide occurring between Alu_{17} and Glu_{18}. When the complete ORF sequence was used to search the non-redundant protein database for similarity, results confirmed the conclusions drawn from the search with the 5′ sequence. Of all sequences returned the highest similarities were to those of the putative and known secreted cellulases (Glycosyl hydrolase family 48), the most significant being that from *Streptomyces coelicolor* (54% similarity over 635 residues). High levels of similarity was also seen to five exocellulases and four endocellulases, all of bacterial origin, with generally more significant similarities towards the exoglucanase classes (48-51% identity in matches compared to 45-49% for the endoglucanases). Searching the Prodomain database with the predicted VW24 product shows greatest similarity to the C-terminal exoglucanase domain of the bi-functional Endoglucanse A precursor of *Caldicellulosiruptor saccharolyticus*. This bi-functional enzyme shows endoglucanase and exoglucanase activity at the N-terminal and C-terminal ends respectively (Leuthi *et al* 1992), VW24 shows similarity to only the exoglucanase domain. The results of the sequence searching shows that three of the five top hits for VW24 are cellobiohydrolase enzymes, and the remaining two are putative or characterised exoglucanases of unknown sub-type. Therefore it is feasible to assume, on the basis of similarity, that VW24 encodes a cellobiohydrolase (CBH) type exoglucanase.

The vine weevil cellulase is considered further in Chapter 6.

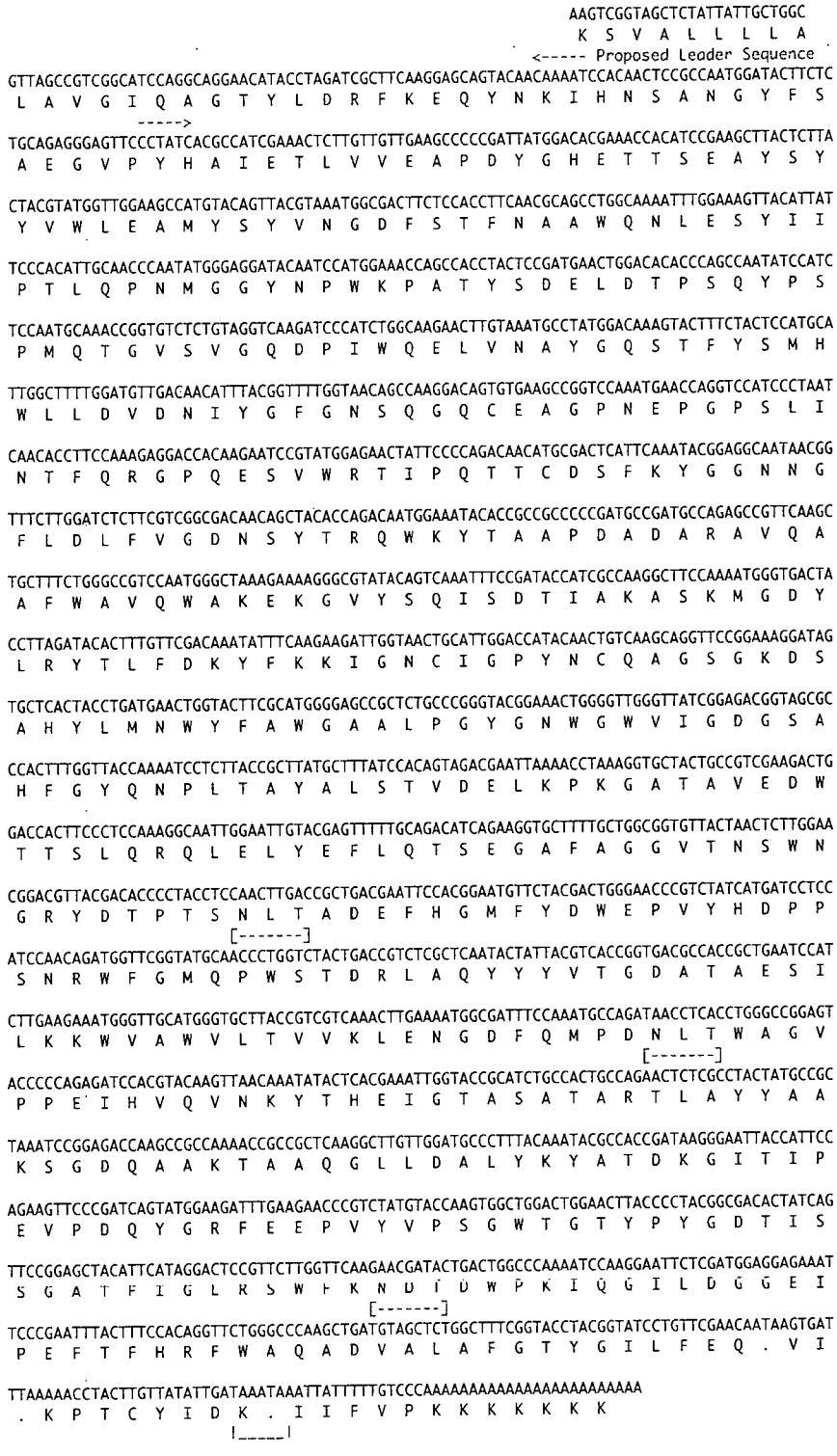


Figure 3-5 Incomplete cDNA sequence of the putative cellobiohydrolase clone VW24 and its predicted translational product, putative N-glycosylation sites ([---]), consensus poly adenylation signal (|__|) and the signal peptide sequence (<-->)

3.7 Discussion

The partial sequencing of a number of cDNA clones from the tissue specific cDNA library gives a rapid indication of the complexity of the mRNA population when the tissues were excised from the host organism, and allows analysis of the predicted proteins present in the organism. This study used total gut tissue from late instar vine weevil larvae as donor material and would be expected to identify proteins associated with general digestive processes, and exclude those with roles involved with mobility and reproduction.

Of the nineteen clones sequenced from the vine weevil gut library five showed significant similarities to known protein products with possible digestive functions (two serine proteases, a pectin methylesterase, a lipase and a cellulase) and therefore may be involved in vine weevil digestion of ingested plant material as food passes along the entire length of the gut. True digestive enzymes need to be secreted from the gut cell wall into the gut lumen where the ingested food is located, this can be seen with known digestive proteases where the enzyme is produced initially as a prepropeptide, and only after removal of the signal peptide and the propeptide is the enzyme located in the gut lumen in an active form. The remaining fourteen clones sequenced were either; deduced to have general house-keeping roles (Elongation factor 2, VW14 and acid phosphatase, VW18. Table 3.2), showed similarity to un-characterised gene products (i.e. VW2, VW25. Table 3.2), or were sequences of low complexity and contained non-coding regions (VW3, VW23. Table 3.2). These clones, several which are believed to be of intracellular origin, were not analysed further in this study.

The presence and significance of two independent trypsin-like protease clones being isolated from the cDNA library will be discussed in Chapter 4.

The putative translation product of VW16 shows homology to the class of enzymes known as pectinesterases, which catalyse the general reaction:

$$\text{Pectin} + n\text{H}_2\text{O} \xrightarrow{\text{Pectinesterase}} \text{Pectate} + n\text{MeOH}$$

The pectinesterase exhibit a strong specificity towards the methyl esters of α-1,4-D-galacturan polymers commonly found as components of the middle lamella of plants cells (Deacon 1984). Figure 3-6 shows the conversion reaction as predicted *in vivo* and indicates the cleavage of the ester bond during hydrolysis. Pairwise alignment of VW16 with several known pectin methylesterase enzymes shows that VW16 shares significant levels of similarity (>35% similarity). Currently the catalytic and structural features of these known pectin methylesterases have not been analysed and are not yet understood. However VW16 does match the pattern of conserved regions seen between other pectinesterases. Although identified by similarity as a pectin methylesterase, such activity is currently not known *in vivo* in the vine weevil. Although the sequence similarity in the appropriate region is not very strong, the vine weevil pectinesterase contains the motif including the putative catalytic tyrosine residue (Markovic and Jornall, 1992). However other pectinesterases where activity has previously been shown (Spok *et al*, 1991, Lauret *et al*, 1993) do not appear to contain either this motif or the conserved tyrosine residue.

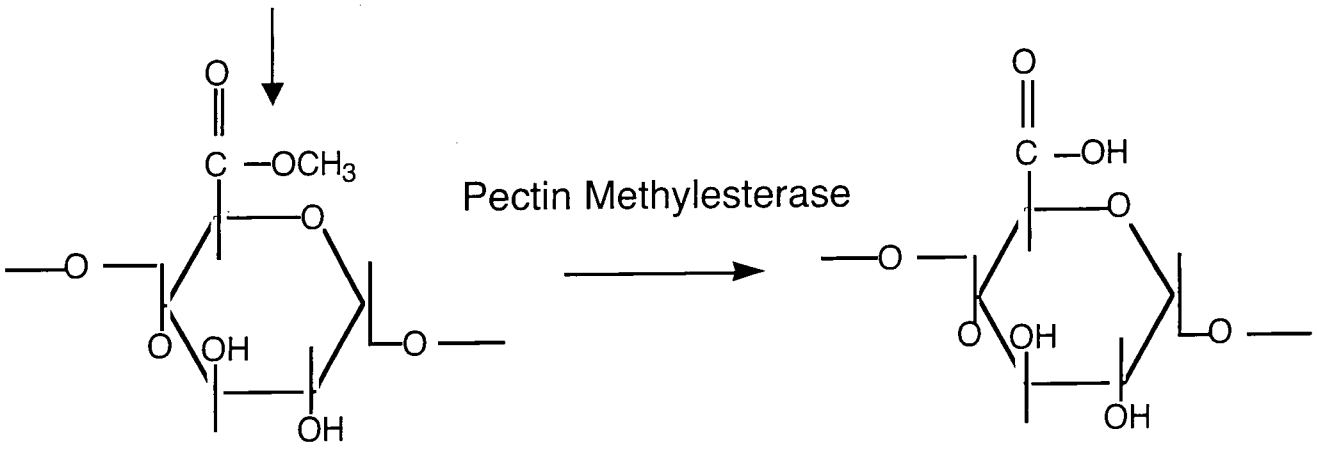


Figure 3-6 Conversion of pectin methyl esters into pectate by pectin methylesterase enzymes. The highlighted arrow indicates ester bond cleaved during hydrolysis.

Once the pectinesterases have converted the pectin methyl esters into pectate this new product subsequently forms a substrate for degradation of the polysaccharide by polygalacturonase enzymes (Deacon 1984). For full digestion of these polysaccharides to occur, the synergistic activity of pectin methylesterase and polygalacturonase enzymes are required. Therefore currently with the isolation of only a pectinesterase from the vine weevil an effective pectin digestive system can only be speculated. However two polygalacturonase transcripts have recently been cloned from the phytophagous beetle *Phaedon cochleariae* (Girard and Jouanin 1999). Both the vine weevil and *P.cochleariae* have a similar taxonomy and dietary intake, and thus the presence of similar enzymes in the vine weevil is likely, giving the vine weevil its own pectic digestive system. The ability of phytophagous insects to digest pectin has largely been unrecognised but is obviously advantageous.

The similarity of the VW16-encoded protein to bacterial pectinesterases raises the question of whether this sequence originates from the insect genome, or has come from the genome of the bacterium in the gut microflora. During isolation of the vine weevil guts, no effort was made to remove gut bacteria or other micro-organisms, and thus RNA (and possible DNA) from these organisms could be present in the cDNA library. However the presence of the polyA-tail seen at the terminal 3' end of the transcript is indicative of a cDNA from eukaryotic origin, thus ruling out contamination from bacterial sequences. It is still possible that the cDNA could have originated from a fungal organism present in the gut, but its greater similarity to bacterial sequences makes this unlikely also. It is reasonable to assume that this is an insect transcript.

The third class of enzyme identified through the random screening of the vine weevil gut library was that of a putative triacylglycerol lipase (VW10), with greatest similarity shown towards Lipase 1 and Lipase 3 of *D.melanogaster*. The high level of similarity seen between these two lipases and VW10 probably shows common ancestry with very little divergence between the transcripts of the two different species. Sequence alignments of VW10 with characterised lipases has shown high levels of conserved sites, especially at predicted active site clusters suggesting that VW10 is a transcript of a functional enzyme. Triacylglycerol lipases as a rule hydrolyse the outer ester links of triacylglycerols by means of a catalytic triad of H, S and D residues orientated in an active site pocket within the enzyme (Noble *et al* 1993). This combination of catalytic residues is chemically analogous to, but structurally different from that seen in the serine protease family of enzymes (Pistillo *et al* 1998). Although the aspartate residue of

the proposed catalytic triad has not been unambiguously identified in VW10, studies have shown that the residue is not essential for lipase activity (Noble *et al* 1993). Further evidence for the activity of the VW10 translational product come from the study of the human storage disease "Walmans disease" where the absence of a conserved leucine residue some twenty six positions to the C-terminal side of the active serine, results in the loss of acid lipase activity (Pistillo *et al* 1998). Analysis of the two *D.melanogaster* sequences and VW10 shows that all three contain this conserved leucine, which would strongly suggest acid lipase activity.

The activity of the triacylglycerol lipases is increased with the addition of surfactants to cause lipid emulsification. Surfactant have not currently been isolated from insects, but the presence of sufficiently high concentrations of surfactant phospholipids (i.e. lysolecthin) in insect midguts could act like mammalian bile salts in reducing the surface tension of the midgut contents (De Veau and Schultz, 1992). The few insect midgut triacylglycerol lipases identified resemble the action and substrate specificity of mammalian pancreatic lipases (Lehane and Billingsley, 1996). Hydrolysis of the triacylglycerol results in the production of 2-monoacylglycerol, which may be absorbed, as seen in *Periplaneta americana* (Dictyoptera: Blattidae) (Bollade *et al*, 1970), or further hydrolysed as with *Locusta migratoria* (Orthoptera: Acrididae) (Weintraub and Tietz, 1973) and *Manduca sexta* (Lepidoptera: Sphingidae) (Tsuchida and Well, 1988).

The exact role of VW10 in the vine weevil can not be determined from this study between that of a secreted digestive acid lipase or a lysosomal acid lipase, therefore its role in the digestion of ingested fatty acids is still uncertain.

The final transcript of interest identified in this study was VW24, a putative cellobiohydrolase (glycosyl hydrolase family 48). Cellobiohydrolases are members of the family of enzymes that degrade cellulose, and are commonly referred to as cellulases. The three major enzymes of the family act synergistically upon insoluble ß-1,4-linked cellulose polysaccharide to degrade it into soluble glucose units. Endo-ß-1,4-glucanases cleave randomly within the polysaccharide chain releasing short chain polysaccharides (cellotriose, cellopentose). Exo-ß-1,4-glucanases (cellobiohydrolase) hydrolyse the glycosidic bond two units from the non-reducing end of microcrystalline cellulose releasing cellobiose as a primary product (Tomme 1995). Finally ß-glucosidase hydrolyses this cellobiose product into single glucose units (Figure 3-7).

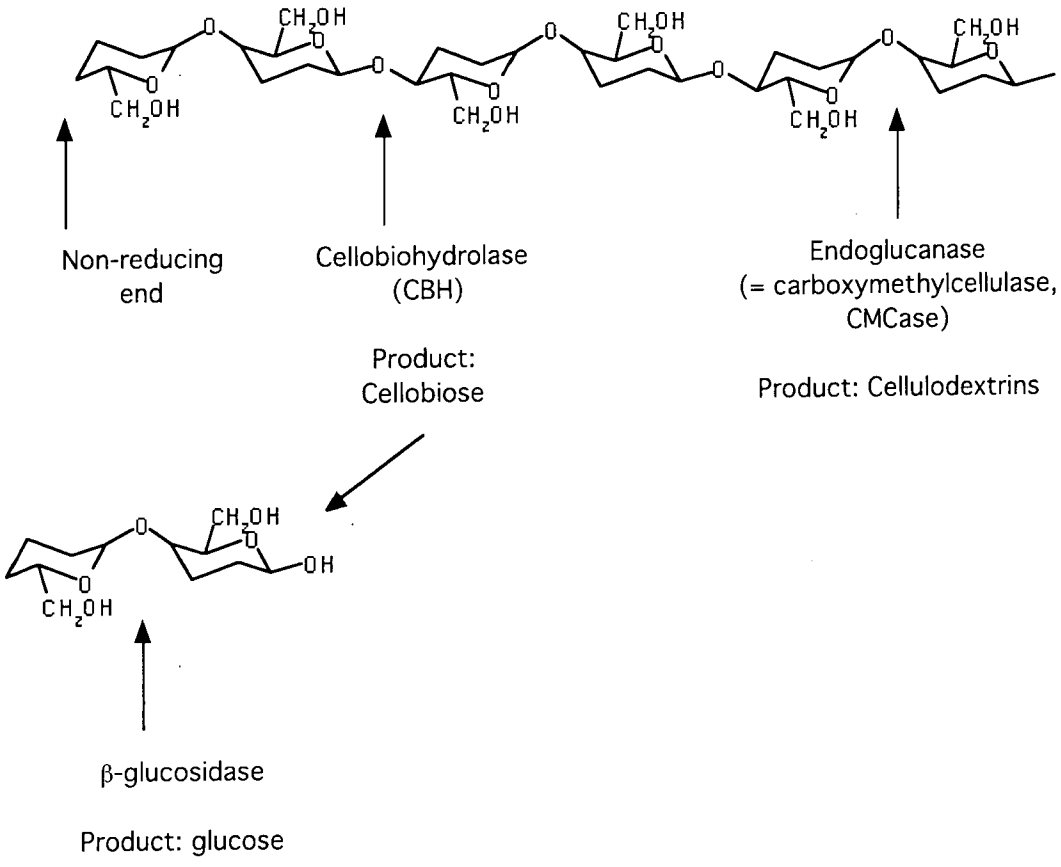


Figure 3-7 Overview of the digestion of crystaline cellulose. CBH attacks microcrystalline cellulose; hydrolyses glycosidic bond 2 in from non-reducing end; usually rate limiting in cellulose degradation. CMCase attacks randomly within cellulose. All three enzymes act synergistically to degrade cellulose

Traditionally cellulases were thought to be of microbial and fungal origin with a few examples isolated in plants, this view has recently been superseded with the discoveries of a putative endogenous endoglucanase cDNA from *Phaedon cochleariae* (Lise Ref) and a genomically encoded endoglucanase from the termite families *Termitidae* and *Rhinotermitidae* (Tokuda *et al*, 1999). The putative translated product of VW24 showed greatest similarity to the cellobiohydrolase of *S.coleocolor* and several other exoglucanases of bacterial origin. Pairwise alignment between these species and VW24 shows regions of conservation throughout the catalytic domain of each enzyme, however the 3′ UTR of VW24 shows all the characteristics of an eukaryotic cDNA with the presence of a polyadenylation signal, and a poly-A tail, which typically differentiates it from the prokaryotic sequences returned from the Blastx similarity searches. No significant similarity was seen towards the endoglucanases from both termite and cockroach, as expected (data not shown). The significance of this clone and its origin will be discussed later in this thesis (Chapter 5).

To conclude, this method of randomly screening the lambda cDNA library is a very efficient method of generating large quantities of data about an organism of interest. The data generated falls into two main categories, those sequences that one would expect (i.e. the identification of protease clones) and those, which one wouldn't, but appear to be more significant (i.e. identification of a cellulase enzyme). However at this time these sequences cannot be definitively classified as endogenous to the vine weevil, due to contaminants of the gut tissue by microbial symbionts and ingested food stuffs. Further more those enzymes with a putative role in digestion, speculated here, may well be vacuole bound and never secreted into the gut lumen.

4 Vine Weevil Gut Proteases

A molecular approach was used to determine which digestive enzymes were present and active in the gut of the vine weevil. A cDNA library, constructed from larval gut tissue, was used as a source of clones encoding digestive proteases. Following the accepted view (Terra *et al*, 1996) that the major proteolytic activity in the gut lumen of coleopteran insects is due to cysteine proteases, the vine weevil cDNA library was initially screened for cDNAs encoding cysteine proteases.

4.1 Generation of a probe for cDNAs encoding cysteine proteases

A sequence corresponding to part of the sequence of a cysteine protease was amplified by RT-PCR from 1μg of poly(A)+ RNA, extracted from the guts of larval vine weevils. The reaction used a generic 3′ primer for the initial reverse transcription, poly$T_{(25)}$ with a single G residue at its 3′ end. Preliminary experiments with T_{25}A, T_{25}C and T_{25}G primers was allow reverse transcription from the 3′ end of all mRNA molecules had shown that the T_{25}G primer gave a specific amplification product whereas the other combination of primer did not. The 5′ primer (5′-TGYGGIIIITGYTGGGCITT-3′) was based on the amino acid sequence CGSCWAF which includes the catalytic cysteine residue and is strongly conserved in cysteine proteases (Barret *et al*, 1998). This primer defined the boundaries for the PCR amplification and predicted a product of 850-1000 bp, depending on the 3′ UTR

Analysis of the RT-PCR products by agarose gel electrophoresis showed the presence of a weak amplification product of approx. 900bp. The PCR product was isolated by excision of the band from gel; DNA was extracted and cloned in pCR2.1 using the TOPO TA cloning method. Five clones, selected at random, were subjected to DNA sequencing to characterise the inserts. Of the five clones, two did not contain any sequence encoding a cysteine protease, whereas the other three clones all contained C-terminal sequences from cysteine proteases. These sequences were compared to a non-redundant global protein database using BLASTP search. One clone was most similar to cathepsin L or S proteases; the other two clones were very similar (although not identical) and corresponded to the C-terminal sequence of cysteine proteases most similar to cathepsin B.

4.2 Screening the vine weevil cDNA library for clones encoding cysteine proteases

The cloned PCR product encoding the C-terminal region of a cathepsin-B-like protease (apparently representing the most abundant type of cysteine protease) was used to screen a cDNA library constructed from gut tissue of vine weevil larvae. The library was constructed in the phage vector λ ZAP II (Stratagene; see methods section). Approx. $5x10^4$ clones from the phage library were plated at a density of 1 plaque/mm^2 on large bioassay plates. The plaques were transferred to nitrocellulose and screened with a ^{32}P-labelled probe generated from the cathepsin-B-like clone using random primer labelling. This screen resulted in the identification of 5 primary plaques which were excised and subjected to plaque purification. Three individual plaques were isolated for each of the primary plaques from this secondary screen. The secondary plaques were excised (*in vivo* excision) to release the pBluescript vector containing the cDNA insert in an *E.coli* host. The resulting recombinant plasmids were isolated and analysed by restriction digestion. Plasmid DNA was digested with PstI and XhoI to release the cloned cDNA insert from the pBlueScript SK vector. Figure 4-1 shows the agarose gel analysis of the digestion products for nine excised clones for each of the original three phage selected. Clones 1a1, 1b1, and 2a2 (corresponding to two primary plaques) were partially sequenced. Analysis of the resulting sequence data showed that all three sequences were the same, putatively corresponding to the same mRNA species.

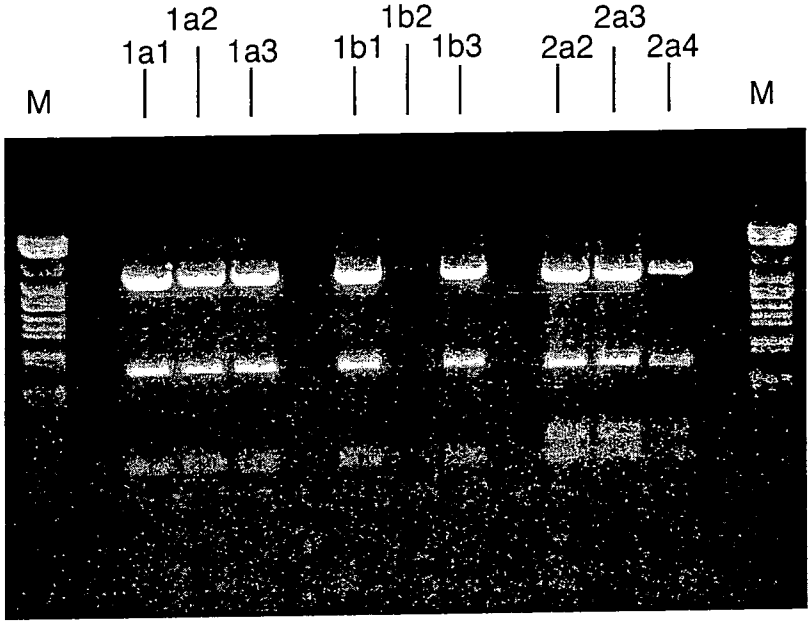


Figure 4-1 Agarose gel showing the nine excised clones corresponding to two primary plaques after digestion with PstI and XhoI to release to cDNA insert. Clones 1a1, 1b1, and 2a2 were selected for partial sequencing.

The cathepsin B-like cDNA was fully sequenced, resulting in the DNA sequence shown in Figure 4.2. The clone contains a full-length coding sequence for a proprotease precursor polypeptide, as judged by similarity to other cathepsin B proteins, but lacks a start codon. The available sequence is thus truncated at the N-terminus, but only insofar as part of a predicted signal peptide (as determined by the SignalP prediction software; www.cbs.dtu.dk/services/SignalP/index.html) is missing. As shown in Figure 4.2, the encoded protein sequence contains a predicted signal peptide of at least 8 residues, a propeptide sequence of 58 residues (based on homology to known cathepsin-B sequences), and a predicted mature protease of 251 amino acids. The mature protein contains sequence features characteristic of cathepsin family cysteine proteases; most importantly, the catalytic triad of residues found in cysteine proteases (Cys_{25} His_{159} Asn_{175}) are present in the correct positions predicted by similarity. A feature characteristic of cathepsin-B proteases, the "occluding loop region" is present at residues 179 and 180, although the expected adjacent histidine residues (His109, 110) are replaced by Asp-His, and the loop is 4 residues shorter than that in other cathepsin-B enzymes.

```
      ATGAAAATTGTTGTAGTATTGAGTGCTTTAATGGCCCTTGCCTTGGCCGAGGGT
       M  K  I  V  V  V  L  S  A  L  M  A  L  A  L  A  E  G
                        Leader Sequence
CCATACAAGGCAGCTTACATACCTTTGGCAACGGAGATTTCGTATGAACCATCACCACGT
 P  Y  K  A  A  Y  I  P  L  A  T  E  I  S  Y  E  P  S  P  R
                        -><-        Pro-Peptide             ->
ATCGTAGGAGGACAGAGTGCTGCCAGAAACCAATTCCCCTACCAAATTTCCCTCCAAAGG
 I  V  G  G  Q  S  A  A  R  N  Q  F  P  Y  Q  I  S  L  Q  R
<-            Mature Protein
CGTGTTGCTACAAACAGTTTCAGCCATATCTGTGGAGGCTCCATTGTTTCTCCTTCCTGG
 R  V  A  T  N  S  F  S  H  I  C  G  G  S  I  V  S  P  S  W

ATACTTACCGCCGCTCACTGCACAGTGCAATTTACTCCTGCCAATCTTAGAGTTGTAGCT
 I  L  T  A  A  H  C  T  V  Q  F  T  P  A  N  L  R  V  V  A
                  ^His 57
GGAATTTTGTTACTTAGCGACCTAAATATCGCCGGGCAACAAATCAGGAATGTTCAGAGT
 G  I  L  L  L  S  D  L  N  I  A  G  Q  Q  I  R  N  V  Q  S

ATCATCAATCATCCATCTTATCCCGGAGGTAATCAGGTAGCACCCAACGACGTCTCTTTA
 I  I  N  H  P  S  Y  P  G  G  N  Q  V  A  P  N  D  V  S  L
                                                ^Asp 102
GTCCGCTTGGCTGCCCTTCTTTCATACACAATCAACGTACAACCTATCGCTATCCCACAA
 V  R  L  A  A  L  L  S  Y  T  I  N  V  Q  P  I  A  I  P  Q

CAAGGACATGTTTCCCGTGGTACTGGTGTCCTCTCTGGATGGGGATTACTACAAACTGGA
 Q  G  H  V  S  R  G  T  G  V  L  S  G  W  G  L  L  Q  T  G

GGTGCCACTCCAAACCATCTTCAATTCGCTAACTTGCCCGTTGTACCTGAAAATGAATGC
 G  A  T  P  N  H  L  Q  F  A  N  L  P  V  V  P  E  N  E  C

AGCGCTCGCTTGACCAGCCTTCTTGGACGTACCACCCCGTTCAGCATTGCCTTGAACCTC
 S  A  R  L  T  S  L  L  G  R  T  T  P  F  S  I  A  L  N  L

TGCTCAGGAACTGTCTTGGGACGCGAATCTGCTTGCAGTGGTGACAGTGGTGGCCCCTAT
 C  S  G  T  V  L  G  R  E  S  A  C  S  G  D  S  G  G  P  Y
                                              ^Ser 195
GTTAAAGATCGCAAAGTTATTGGAGTCGTTTCCTGGGGACTTACTCCATGTGGTAATTCA
 V  K  D  R  K  V  I  G  V  V  S  W  G  L  T  P  C  G  N  S

GGAGCTCCTTCAGTNTACGTCAAGACTTCTGCCTATACCAGCTGGATCACTACCAACACT
 G  A  P  S  V  Y  V  K  T  S  A  Y  T  S  W  I  T  T  N  T

AACGGAGAAGTTAGACCTTAAAATGCTTATGAACAAATATACTTAATTAATAAAATAAAT
 N  G  E  V  R  P  .  N  A  Y  E  Q  I  Y  L  I  N  K  I  N

TCTTCTGTACATTTAAAAAAAAAAAAAAAAAAA
 S  S  V  H  L  K  K  K  K  K  K
```

Figure 4-2 Consensus sequence summary of the trypsin-like cDNA clones (VW5 and VW11) isolated from the vine weevil gut specific cDNA library. The catalytic triad residues have been identified (His 57, Asp 102, and Ser 195) through similarity.

4.3 Isolation of cDNA clones encoding serine proteases

The vine weevil larval gut cDNA library was also analysed by random sequencing of clones. $1x10^7$ individual phage were subjected to mass excision and resulting bacteria containing recombinant pBluescript plasmids were plated out. 25 bacterial colonies were chosen at random, and plasmids were purified from them. Plasmids were analysed by restriction digestion as above, and those containing inserts of >500bp were selected for DNA sequencing. From those clones sequenced, two contained inserts encoding proteins similar to serine proteases, as judged by a search of the non-redundant protein database using BlastX (see Chapter 3 for description of the remaining clones). Complete sequencing of these two clones, VW5 and 11, showed that VW11 contained a putatively full-length coding sequence, whereas VW5 was truncated by only a few amino acids at the N-terminus. As shown in Figure 4.3, both clones contained a predicted signal peptide, a short pro-peptide, and the normal catalytic triad of residues His_{57} Asp_{102} Ser_{195} in the expected positions in the mature protein when compared with the consensus serine protease sequence.

```
      ATGAAAATTGTTGTAGTATTGAGTGCTTTAATGGCCCTTGCCTTGGCCGAGGGT
       M  K  I  V  V  V  L  S  A  L  M  A  L  A  L  A  E  G
                        Leader Sequence
CCATACAAGGCAGCTTACATACCTTTGGCAACGGAGATTTCGTATGAACCATCACCACGT
 P  Y  K  A  A  Y  I  P  L  A  T  E  I  S  Y  E  P  S  P  R
                         ->><-        Pro-Peptide            ->
ATCGTAGGAGGACAGAGTGCTGCCAGAAACCAATTCCCCTACCAAATTTCCCTCCAAAGG
 I  V  G  G  Q  S  A  A  R  N  Q  F  P  Y  Q  I  S  L  Q  R
<-            Mature Protein
CGTGTTGCTACAAACAGTTTCAGCCATATCTGTGGAGGCTCCATTGTTTCTCCTTCCTGG
 R  V  A  T  N  S  F  S  H  I  C  G  G  S  I  V  S  P  S  W

ATACTTACCGCCGCTCACTGCACAGTGCAATTTACTCCTGCCAATCTTAGAGTTGTAGCT
 I  L  T  A  A  H  C  T  V  Q  F  T  P  A  N  L  R  V  V  A
                ^His 57
GGAATTTTGTTACTTAGCGACCTAAATATCGCCGGGCAACAAATCAGGAATGTTCAGAGT
 G  I  L  L  L  S  D  L  N  I  A  G  Q  Q  I  R  N  V  Q  S

ATCATCAATCATCCATCTTATCCCGGAGGTAATCAGGTAGCACCCAACGACGTCTCTTTA
 I  I  N  H  P  S  Y  P  G  G  N  Q  V  A  P  N  D  V  S  L
                                                 ^Asp 102
GTCCGCTTGGCTGCCCTTCTTTCATACACAATCAACGTACAACCTATCGCTATCCCACAA
 V  R  L  A  A  L  L  S  Y  T  I  N  V  Q  P  I  A  I  P  Q

CAAGGACATGTTTCCCGTGGTACTGGTGTCCTCTCTGGATGGGGATTACTACAAACTGGA
 Q  G  H  V  S  R  G  T  G  V  L  S  G  W  G  L  L  Q  T  G

GGTGCCACTCCAAACCATCTTCAATTCGCTAACTTGCCCGTTGTACCTGAAAATGAATGC
 G  A  T  P  N  H  L  Q  F  A  N  L  P  V  V  P  E  N  E  C

AGCGCTCGCTTGACCAGCCTTCTTGGACGTACCACCCCGTTCAGCATTGCCTTGAACCTC
 S  A  R  L  T  S  L  L  G  R  T  T  P  F  S  I  A  L  N  L

TGCTCAGGAACTGTCTTGGGACGCGAATCTGCTTGCAGTGGTGACAGTGGTGGCCCCTAT
 C  S  G  T  V  L  G  R  E  S  A  C  S  G  D  S  G  G  P  Y
                                                 ^Ser 195
GTTAAAGATCGCAAAGTTATTGGAGTCGTTTCCTGGGGACTTACTCCATGTGGTAATTCA
 V  K  D  R  K  V  I  G  V  V  S  W  G  L  T  P  C  G  N  S

GGAGCTCCTTCAGTNTACGTCAAGACTTCTGCCTATACCAGCTGGATCACTACCAACACT
 G  A  P  S  V  Y  V  K  T  S  A  Y  T  S  W  I  T  T  N  T

AACGGAGAAGTTAGACCTTAAAATGCTTATGAACAAATATACTTAATTAATAAAATAAAT
 N  G  E  V  R  P  .  N  A  Y  E  Q  I  Y  L  I  N  K  I  N

TCTTCTGTACATTTAAAAAAAAAAAAAAAAAAA
 S  S  V  H  L  K  K  K  K  K  K
```

Figure 4-3 Consensus sequence summary of the trypsin-like cDNA clones (VW5 and VW11) isolated from the vine weevil gut specific cDNA library. The catalytic triad residues have been identified (His 57, Asp 102, and Ser 195) through similarity.

4.4 Characterisation of proteolytic activity within the vine weevil gut

The isolation of cDNA clones encoding two classes of proteolytic enzyme from the vine weevil larval gut cDNA library suggested that digestive proteolysis in this insect may differ from that in other coleopteran species. Biochemical characterisation of proteolytic activity within the gut lumen of larvae vine weevils was carried out using a general fluorescent protease substrate (casein-fluorescein, BODIPY FL). A pH curve of proteolytic activity in the larval gut extract (Figure 4-4) showed low activity in the acidic pH region (3.5 – 6.5), with an ill-defined slight peak in the region 4.5-6.5, but an increasing activity over the alkaline region (8-10.5). This result is consistent with the presence of both cysteine proteases (active at acidic pH) and serine proteases (active at alkaline pH).

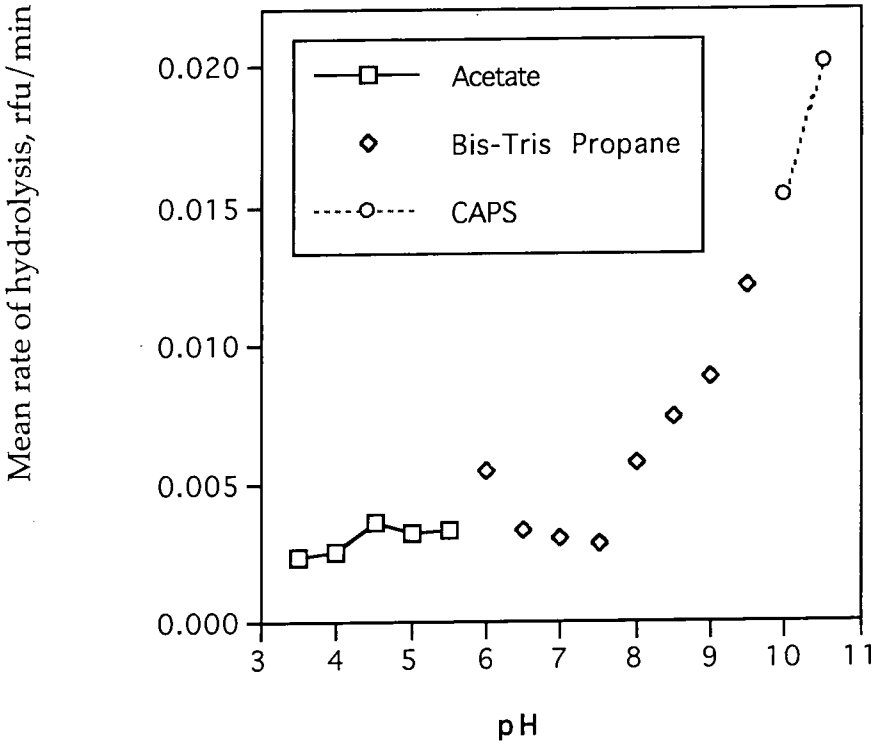


Figure 4-4 pH profile for protease activity in vine weevil larval gut crude extracts (EnzCheck Substrate) showing mean rate of hydrolysis between n samples, where n=3.

To determine precisely which enzyme class was responsible for each peak of activity specific enzyme inhibitors were included in the assay at both an acidic pH (4.5) and an alkaline pH (9.5). The chemical inhibitor E64 was used at 10μM to inhibit cysteine protease activity, and the protein protease inhibitor soybean Kunitz trypsin inhibitor (SKTI) was used at 1μM to inhibit serine proteases. Figure 4-5 shows the effect of adding each protease inhibitor at the two different pH values has on the proteolytic activity of crude gut extract. When proteolysis was determined at pH 4.5 hydrolysis of the casein substrate is reduced by 74% by the addition of 10μM E64, and by 30% by the addition of 1μM SKTI. The addition of both the inhibitors to the reaction at pH 4.5 reduced substrate hydrolysis by over 85% as compared to the uninhibited reaction.

Inhibition of gut proteases at pH 9.5 was also seen with E64 and SKTI (Figure 4-5), with the trypsin inhibitor (SKTI) inhibiting 93% of proteolytic activity and E64 only inhibiting 14% of enzyme activity. The use of both E64 and SKTI in assays performed at pH 9.5 gave only very slight increases in inhibition when compared to SKTI alone.

When used in tandem these protease did not give 100% inhibition of the substrate hydrolysis, this may be due to the presence of other inhibitor insensitive protease being present within the gut lumen or the associated tissue. By using a range of additional inhibitors it should be possible to identify which other classes of protease are present and to what degree they contribute to the overall digestive capability of this insect.

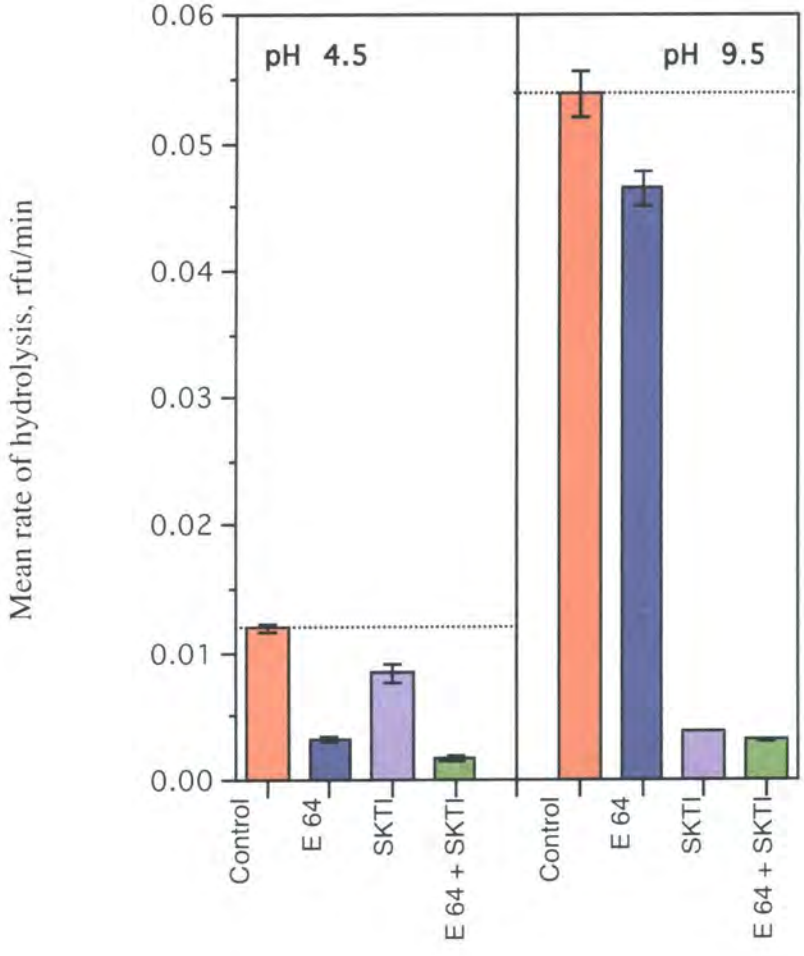


Figure 4-5 Vine weevil gut protease inhibition assays using EnzCheck substrate. Assays performed at pH 4.5 and pH 9.5 with control; gut extract + substrate, E64; gut extract + substrate + E64 (10μM), SKTI; gut extract + substrate + SKTI (1μM), E64 + SKTI; gut extract + substrate + E64 and SKTI. Points and bars represent the mean ± the standard deviation across triplicate samples.

4.5 Discussion

The ability of phytophagous insects to utilise the relatively low nitrogen content of plant tissues is typically the limiting factor in their nutritional uptake. In the larval stage, the vine weevil feeds predominantly on root tissues of plants, chewing and consuming fairly indiscriminately. The root tissue as a whole has low levels of free amino acids, and thus effective hydrolysis of dietary proteins is essential for survival. This study shows how proteolytic enzymes from two mechanistic classes, cysteine and serine proteases, have been identified and characterised in the gut of larval vine weevil.

4.5.1 Biochemical Characterisation of Protease Activity

Following the characterisation of gut proteases in several phytophagous coleopteran insects (reviewed by Terra *et al*, 1996), it has become an accepted view that Coleopteran insects have acidic conditions in the midgut, and use cysteine proteases as the predominant enzymes for protein digestion. A previously published study (Michaud *et al*, 1995) provided evidence that this was the case in vine weevil also. Gut extracts from adult vine weevil showed maximal proteolytic activity towards protein substrates at pH 5.0, with a second peak (approx. 60% of maximum) at pH 9.0. Assays carried out at pH 5.0 indicated that only cysteine protease inhibitors (E-64 and oryzacystatin) had any significant effect on protease activity, while inhibitors of serine proteases (PMSF, AEBSF and SKTI) or aspartic proteases (pepstatin) had no effect. However, a significant proportion of protease activity (approx. 40%) was not inhibited by any of the inhibitors tested. Inhibition studies were not carried out at alkaline pH . The present results appear to be in disagreement with these earlier data, but were obtained with gut extracts from larval rather than adult vine weevils. With the larval gut extract, hydrolysis of a protein substrate was much more active under alkaline conditions, and only low levels of proteolysis at acidic pH were observed. The biochemical data do suggest that some cysteine proteases are active within the larval vine weevil gut, since in assays carried out at pH 4.5 the specific cysteine protease inhibitor E-64 reduces proteolysis by 74%. However, even at pH 4.5 the serine protease inhibitor SKTI reduced levels of substrate hydrolysis by approx. 30%, and appox. 15% of proteolysis was not inhibitable by either E-64 or SKTI, suggesting that proteolysis under these conditions could not be attributed solely to cysteine proteases.

Assays carried out at pH 9.5 gave unambiguous evidence for the presence of serine proteases as major digestive enzymes in the larval vine weevil gut. These conditions are non-optimal for cysteine protease activity, and as expected, proteolysis at this pH was reduced only slightly (<10% inhibition) by the addition of E-64. On the other hand, approximately 85% of the proteolytic activity could be inhibited by the addition of the serine protease inhibitor SKTI, and thus at this pH serine proteases are predominant. The pH curve for protein hydrolysis shows that the mean rates of total hydrolysis at pH 9.5 are approximately 5 times greater than those at pH 4.5, suggesting that the serine proteases are likely to represent the majority of digestive protease activity. In their study of adult vine weevils, Michaud *et al* (1995) did not characterise the protease activity they observed under alkaline conditions, and thus may have overlooked the presence of serine proteases in their gut extracts. The combined data suggest that vine weevil gut proteolysis shifts from predominantly serine protease-based to predominantly cysteine protease-based as the insects develop from larvae to adults. The larval stage of this pest is more damaging to plants, due to greater consumption of plant material, and feed from roots, as opposed to the adult stage which feeds predominantly from leaves. The change in digestive protease composition may thus be a result of changes in feeding requirements and habit. Although the presence of serine proteases in this coleopteran species is not as expected, other phytophagous coleopterans have been found to possess and alkaline pH optimum for gut proteolysis, and have been shown to contain digestive serine proteases; these species include cabbage seed weevil (Girard *et al*, 1998), *Baris coerulescens* (Bonade-Bottino *et al*, 1999) and red palm weevil (Alarcon *et al*, 2002).

4.5.2 Cysteine Proteases

Isolation of cDNA clones encoding putative digestive enzymes suggested that mRNAs encoding cysteine proteases were less abundant than those encoding serine proteases in gut tissues, in agreement with the biochemical data. Screening the gut cDNA library for cathepsin-B-like enzymes gave only 5 positive plaques from some $5x10^4$ λ phage plague forming units (pfu), indicating a low abundance. In contrast, the two trypsin-like clones were identified by randomly selecting and sequencing 19 excised phagemids, when no additional cysteine protease-like clones were identified in this way. The relative abundance of the trypsin-like clones is thus potentially several orders of magnitude greater than that of the cathepsin-B-like clone.

The cathepsin B-like sequence isolated from the vine weevil gut cDNA library has several features of interest. Most notable is the replacement of the conserved his-his sequence in the "occluding loop" region of the enzyme which is involved in substrate specificity and constraining this enzyme to act as a dipeptidyl peptidase with an asp-his motif. An extensive search of sequences of enzymes with similarity to cathepsin B showed that some variability in the first residue of the his-his sequence is tolerated, with cathepsin B-like enzymes from nematodes showing asn (*Necator americanus*; accession AJ132421) or ala (*Haemonchus contortus*; accession AF046229) at this position, and a cathepsin B enzyme from a platyhelminth (*Fasciola hepatica*; accession AAD11445) having an asp-his sequence similar to the vine weevil enzyme. However, all the insect cathepsin B-like enzymes so far characterised (*Bombyx mori*, silkworm, BAB40804, *Helicoverpa armigera*, corn earworm, AAF35867, *Sarcophaga peregrina*, flesh fly, S38939, *Glossinia marsitans*, dipteran, AF329480) have the sequence his-his present. A cathepsin B-like enzyme in the coleopteran *Apriona germani* (AF483623) has the occluding loop region largely missing and may not be a true cathepsin B. The *Drosophila* genome contains only one true cathepsin B sequence, CG10992, which has the his-his sequence. A second gene has similarity to cathepsin B (CG3074), but the occluding loop region is largely missing. The occluding loop region in the vine weevil cathepsin B enzyme itself has a deletion of 4 amino acid residues when compared to the consensus cathepsin B sequence, and all the other insect cathepsin B enzymes; this deletion is located 2 residues C-terminal to the his-his sequence. The "occluding loop" region, and the his-his residues in particular, are thought to act as a steric block to the active site of cathepsin B, preventing peptide substrates which extend beyond the S_2 sub-site 2 residues N-terminal to the cleaved bond being accomodated. By preventing long peptide substrates from being "threaded" through the active site, the occluding loop thus constrains the enzyme to act as a dipeptidyl peptidase. The replacement of a (potentially) positively charged histidine residue with a negative aspartate residue, coupled with an adjacent deletion of 4 residues, would be expected to significantly alter the conformation of this region of the protein, and may thus affect the substrate specificity of this enzyme compared to other cathepsin B enzymes. The substrate specificity of vine weevil cathepsin B could not be investigated in the present work, but could be addressed through production of the protein by a heterologous expression system. The larval vine weevil gut clearly contains other cysteine proteases, as shown by the initial clones of PCR products amplified by generic cysteine protease primers, and possibly these include a more "conventional" cathepsin B.

The vine weevil cathepsin B sequence contains two potential N-glycosylation sites, as predicted by the presence of the motif N-X-(S/T); these are at amino acids 118-121 (NGT) and 163-166 (NNT). In combination with the presence of a signal peptide, these sites suggest that the enzyme enters the secretory pathway on synthesis, but do not establish whether it is an extracellular (and thus digestive) or intracellular (lysosomal) enzyme. Mammalian cathepsin B enzymes, which are targetted to the lysosome, contain conserved N-X-S/T glycosylation motifs, giving two sites of potential N-glycosylation, one in the pro-sequence, and one in the occluding loop region of the mature enzyme (Mort, 1998). The carbohydrate side chain at the latter site is known to carry the mannose-6-phosphate residue which directs the enzyme to the lysosome (Mach *et al*, 1992). While lysosomal targetting in mammalian cathepsins can be predicted by the presence of glycosylation sites plus the presence of lysine-based structures (Cuozzo *et al*, 1998), the lysosomal transport system in insects does not utilise the mannose-6-phosphate receptor mediated pathway (Aeed and Elhammer, 1994), and the sequences of cathepsin B-like enzymes from invertebrates do not show the conserved potential glycosylation sites observed in mammalian enzymes (data not presented). The vine weevil cathepsin B C-terminal region shows limited similarity to the netrin C-terminal domain (InterPro IPR001134), which is characteristic of some extracellular proteins in mammals, but this similarity is unlikely to be significant, since the mammalian proteins have functional roles in cell development and not in digestion. Prediction of a cellular location for the vine weevil enzyme on the basis of sequence data is thus not possible, and whether the clone characterised here in fact encodes the enzyme responsible for the cysteine protease activity observed in the larval gut extract remains to be established.

4.5.3 Serine Proteases

Two cDNA sequences encoding trypsin-like enzymes were identified during a random sequencing experiment where 19 individual clones excised from the gut cDNA library were analysed, whereas no cathepsin-B-like sequences were identified. The apparent abundance of the trypsin-like cDNA clones (2/19) suggests that these enzymes are being actively synthesised in the larval gut, since their mRNAs are present as a relatively high proportion of the total complement of mRNA species. The trypsin-like enzymes are thus likely to be present as a high proportion of the total extracted gut proteins, whereas the cathepsin B enzyme is probably present at lower levels (mRNAs encoding this enzyme are present as a fairly low proportion of the gut cDNA library, as shown by the library screening experiment). This deduction is in agreement with

conclusions drawn from the biochemical analysis, that serine protease activity made up the major proportion of total gut proteolytic activity, with cysteine protease activity a comparatively minor component.

Both encoded trypsin-like enzymes contain signal peptides; that of VW11 appears to be complete, with the first met residue indicated as start codon; signal peptide cleavage is predicted to take place between residues 16 and 17. The signal peptide in VW5 is clearly incomplete, as the start codon is missing, but analysis using SignalP suggests that only a few amino acids have been truncated, as the polar N-terminal region is present. The predicted cleavage point is between residues 15 and 16. Both enzymes have the consensus N-terminal sequence for mature trypsin, IVGG- approximately 20 residues C-terminal to the signal peptide cleavage point, preceded immediately by an arginine residue. Consequently, sequence analysis predicts that both enzymes contain a propeptide (of 21 and 22 residues in VW5 and VW11 respectively), which is removed by proteolytic cleavage by an enzyme with trypsin-like specificity. There is an N-glycosylation site (–NLT–) in the propeptide of VW5, but not in VW11; this is the only potential glycosylation site in either enzyme.

The active site residues of VW5 map well onto the consensus sequence for trypsins (based on mammalian enzymes). The active site serine (residue 242; equivalent to serine 195 in bovine trypsin) is preceded by an aspartate residue, and is a strongly conserved region of sequence. The predicted substrate pocket specificity determining residue is asp237 (equivalent to aspartate 189), predicting that the enzyme will show trypsin-like specificty towards amino acids with basic side chains. There is a 4 amino acid deletion between the catalytic serine residue and the substrate pocket region, but sequence over this region is well conserved, and gly259 and gly269 can be recognised as the equivalents of gly216 and gly226 in bovine trypsin, which allow access of bulky side chains to the substrate binding pocket. Analysis of the sequence suggests that the enzyme encoded by VW5 is a typical trypsin-like serine protease, and it is likely that this sequence corresponds to a digestive protease. The 50 most similar sequences to VW5 as determined by a BLASTP similarity search are all insect or arthropod trypsins, with one sequence (accession AAC25596) signficantly more similar than all the others (similarity score 8e-68; next best match *Drosophila melanogaster* CG3229 gene product, similarity score 2e-46). The best match sequence is a trypsin from a coleopteran species, the citrus weevil *Diaprepes abbreviatus*. (Yan *et al*, 1999).

On the other hand, although the enzyme encoded by VW11 has sequence homology to trypsins, the active site residues suggest that this is not a trypsin-like protease. The catalytic serine is ser234, and once again the sequence around this serine is sufficiently well-conserved to make its identification unambiguous. The residue at the bottom of the substrate binding pocket, at position 238, is a serine, not an aspartate. Both mammalian chymotrypsin and elastase proteases have a serine residue in this position, in order to accommodate neutral amino acid side chains, and the mutation of the corresponding aspartate residue (asp189) in bovine trypsin to a serine abolishes the specificity of the enzyme towards basic amino acids (Graf *et al*,1988). The sequence over the binding pocket region of VW11 is not well conserved compared to the consensus trypsin sequence, making identification of residues at the top of the binding pocket difficult, but if cys255 is assumed to correspond to cys219 in trypsin (which is involved in a disulphide bond and is strongly conserved) then the residues are leucine 252 and serine 262. These are both bulky residues, which would restrict access to the pocket, and suggest the enzyme might have a specificity similar to elastase. The 6 most similar sequences to VW11, as determined by a BLASTP similarity search of the total protein database, are insect serine proteases, with individual proteins described as being either trypsins or chymotrypsins. The VW11 sequence shows the highest similarity to a partial cDNA sequence (accession no. AF377980) from another Coleopteran, *Anthonomus grandis* (cotton boll weevil), which is described as a trypsin but, like VW11, has a serine at the equivalent position to asp189 in bovine trypsin, and thus cannot have trypsin-like proteolytic cleavage specificity. The sequence analysis would thus conclude that VW11 encodes a protease with chymotrypsin or elastase-like specificity, which is likely to function in a digestive role. This would not be inconsistent with the inhibition of proteolysis observed when assays are carried out in the presence of SKTI, since this inhibitor is effective against chymotrypsin and trypsin, although the inhibition constants for chymotrypsin show that binding to this enzyme is less strong, and has some inhibitory effect on elastases.

The presence of both cysteine and serine protease activity in the crude gut extract, and the identification of cDNA clones encoding proteases of both mechanistic classes suggests that the vine weevil possesses the ability to use cysteine and serine like proteases for dietary proteolysis. This has been seen with *Helicoverpa armigera* (Patankar *et al* 2001), as well as in other coleopteran species, in particularly with the water rice weevil (*L. suffrian*) from which a complex proteolytic system that includes cathepsin D-, cathepsin B-, trypsin-, and chymotrypsin-like activities has been identified (Hernandez

et al, 2003). Hernandez *et al* also show that there is a spatial distribution of the enzymes within the weevil gut with the trypsin-like activity being found evenly among the anterior, middle and posterior portions of the gut, while the thiol proteases activity was most abundant in the anterior and middle gut sections, and the chymotrypsin-like activity was highest in the middle and posterior sections.

The presence of serine protease activity as the major component of digestive proteolysis in vine weevil larvae is in agreement with evidence presented by Gordon *et al* (1997), who exposed newly hatched vine weevil larvae to transgenic strawberry plants expressing the cowpea trypsin inhibitor (CpTI). This Bowman-Birk type protein serine protease inhibitor had previously been shown to be antimetabolic towards a range of insect pests including *Heliothis* and *Spodoptera* when incorporated into artificial diets (Hilder, 1987), and had similar effects on lepidopteran larvae when expressed in transgenic tobacco and potato plants (Hilder *et al*, 1987). Expression of CpTI in strawberry plants reduced feeding damage caused by the vine weevil larvae with a 2-4-fold increase in root weight of the best transgenic lines compared with the controls. This result would be difficult to explain if vine weevil used cysteine proteases as major digestive enzymes, since CpTI has no inhibitory activity towards these enzymes. However, the antimetabolic effect observed in transgenic strawberry is consistent with the use of serine proteases as major digestive enzymes by the vine weevil larvae.

5 Vine Weevil Gut Chitinase

Chitinases are enzymes that specifically hydrolyse the insoluble structural polysaccharide chitin (where n≥11), the enzyme acts upon the ß-1,4-linked *N*-acetylglucosamine of the substrate. In arthropods chitinases have been isolated from the moulting fluids, venom glands and midguts of several different orders. The *in vivo* role of enzyme is believed to be linked with the need for insect to periodically shed their old exoskeletons and continuously or periodically shed their peritrophic membranes and re-synthesise new ones (Lehane 1997).

No chitinases were obtained from the random screen and therefore a clone encoding chitinase was obtained by PCR.

5.1 Cloning of a Chitinase Fragment by 5'RACE

Sequence alignment of several known chitinolytic enzymes from different orders of organisms showed many conserved regions of homology throughout the full length of the peptide sequence. Of these conserved regions, one region of eleven amino acids had been identified that shares >90% homology between all sequences analysed (Kramer, 1997) FDG[L/F/V]DLDWEYP. This region has been identified as the active site for chitinolytic activity. Using this information a degenerate anti-sense primer was designed towards this region (5'-GGRTAYTCCCARTCXAXRTCXAXXCCRTCRAA-3') with the aim to isolate the N-terminal fragment of a chitinase cDNA from larval vine weevil.

Using 5'RACE ready vine weevil gut cDNA as a template for PCR, the fragment of a chitinase cDNA was amplified under standard conditions using the degenerate chitinase active site primer and the universal primer mix (supplied in 5'RACE kit, Clontech). Following PCR, the product was visualised to verify it corresponded to the expected size (by homology to *M.sexta*) of between 600-700bp (Figure 5-1). After purification, the PCR product was TA cloned in the usual manner. The insert size of 10 colonies was analysed by digestion of the isolated plasmids with the restriction enzyme *EcoRI*, after which clone 5'Chitinase2 was partially sequenced using the M13 reverse sequencing site of pCR2.1.

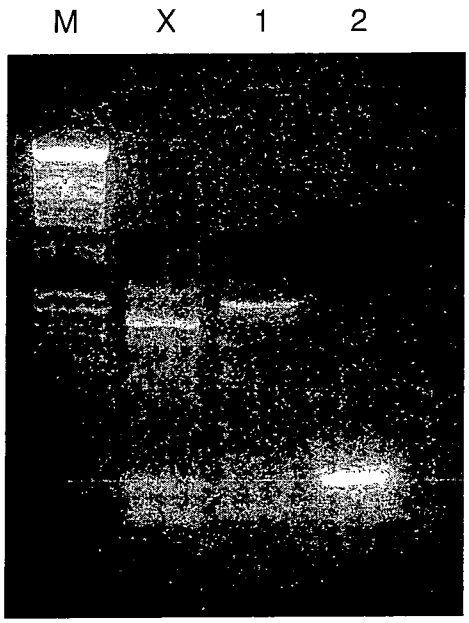


Figure 5-1 Agarose gel (1%) showing the product generated by the 5' RACE performed on vine weevil larval gut mRNA using the chitinase active site primer and the universal primer supplied by Clontech. Lane M; Eco471 molecular weight marker, lane 1; chitinase amplification product, lane 2; no cDNA control. (Lane X not relevant in this instance).

After removal of the cloning vector and the region corresponding to the SMARTII oligonucleotide at the ends of the sequence, a putative ORF was identified starting at nucleotide position 111 and running through to position 551, where the degenerate active site primer is located without any obvious stop signals. Preceding the putative start codon (ATG) at position 111 is a 5'UTR of 110 nucleotides containing 6 stop codons. The ORF sequence for 5'Chitinase2 was compared to the Blastx database to allow it to be identified; of the results returned the top ten hits all were of characterised or putative chitinases, with several coming from an insect origin (*D.melanogaster, B.mori* and *M.sexta*).

5.2 Isolation of a Vine Weevil Chitinase cDNA

The identification of 5'Chitinase2 as a fragment of a true chitinase would allow the clone to be used to probe the previously constructed lambda ZAP cDNA vine weevil gut library. After three sequential rounds of screening with the ^{32}P-dCTP labelled probe (generated from the 5' RACE PCR product) a distinct double positive plaque was isolated. The recombinant pBlueScript plasmid was isolated from the excised phage and

the cloned insert was sequenced in both directions from the SK and T7 sequencing sites within the MCS of the vector to give two partial sequences from the 5′ and 3′ ends of the cDNA insert. Sequence analysis using Sequencher 4.1 showed that the central region of the 5′ partial sequence was identical to that of the probe (5′Chitinase2) but did not contain the first 154 bases of the ORF and the 5′UTR region. This 5′ sequencing event did however extend a further 485 bases downstream of the sequence gained from the 5′RACE. The partial 3′ sequence shows the presence of a polyA-tail of 20 consecutive adenine bases, preceded by a consensus poly adenylation signal (AATAAA) starting 19 bases upstream of the polyA-tail. A further 137 bases upstream a consensus stop codon (TAA) was identified. The two partial sequences did not align to give an overlapping contig, further restriction analysis with *PstI* and *XhoI* indicated that the clone was 1550bp in length (data not shown), the two sequencing runs totalled 1458bp suggesting that some 92 bases of unsequenced material remained in the centre of the clone. These unsequenced bases were identified by digesting the pBlueScript Chitinase cDNA clone with *NcoI* (cuts once within the cDNA clone) and *XbaI* (which cuts within the SK primer region of the pBlueScript MCS), allowing the vector to be re-ligated, without the known 5′ region of the chitinase clone, and subsequently sub-cloned. Sequencing of the sub-cloned vector provided sequence data that covered the previously unknown region, and once aligned with the earlier two sequencing events a contiguous sequence of 1797bp was constructed. The contig shows the presence of an open reading frame from position 113 to position 1622 (1509bp).

BlastP searching with the translational product of the total vine weevil chitinase clone reiterated the initial results gained from similar searching with the 5′RACE fragment and thus confirms this clone as a cDNA encoding a chitinase.

Alignments between the vine weevil chitinase and chitinases from *A.gambiae, Chelonus sp.* and *P.cochleariae* show several conserved amino acid residues throughout the entire sequence (Figure 5-2). Several of these making sequential runs of conserved consensus sequences. Two such regions have been identified in many chitinolytic enzymes; GGWN[A/E]G[S/T] and FDG[L/F/V]DLDWEYP with the latter containing the active residues and defining the catalytic region of the enzyme. A second region found in chitinases is termed the PEST (Kramer, 1997) region due to the large percentage content of proline, glutamate, serine and threonine residues. However is was noted that this region had been replaced by a serine, aspartate and threonine rich region, containing six SSSSDT and one ASSSDT repeated sequences. The typical PEST region is followed by a

cysteine rich domain at the C-terminal end of the sequence, thought to act as a chitin-binding domain. This region can be identified within the vine weevil clone, where six cysteine residues are located.

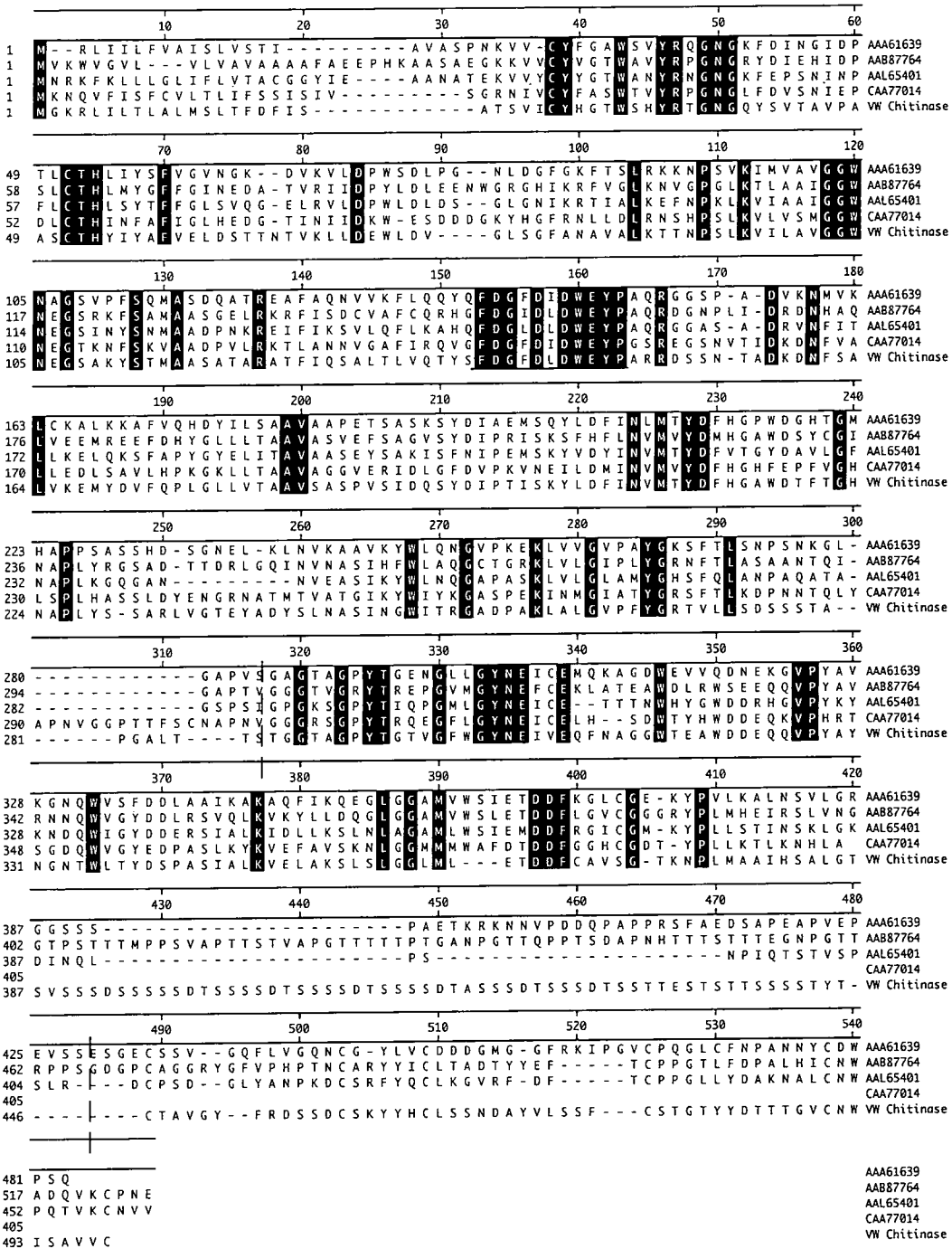


Figure 5-2 Multiple alignments of the predicted protein product from the isolated vine weevil (VW) chitinase cDNA and other known chitinases. Dark shading indicates where residues are fully conserved with the consensus. AAB87764; *Anopheles gambiae*, chitinase, AAA61639; *Chelonus sp.*, venom chitinase, AAL65401; *Glossina morsitans morsitans*, chitinase Chit1 precursor, CAA77014; *Phaedon cochleariae*, chitinase. The underlined region (___) signifies the active site region and vertical bars (|) indicate the boundaries of the conserved domains (catalytic domain | "PEST"/"SDT" domain | conserved cysteine domain).

5.3 Expression of the Vine Weevil Chitinase in Tobacco for use as a Biopesticide

As previously described many insects produce chitinases as gut enzymes to facilitate continual resynthesis of their peritrophic matrices. Expression of insect chitinases in transgenic plants has been proposed as a method for plant protection against insect pests (Kramer, 1997). This pesticidal activity is proposed to act by disrupting the balance of chitinase activity within an insect gut thus effecting the integrity of the peritrophic matrix.

5.3.1 Cloning the Vine Weevil Chitinase into a Plant Expression Vector

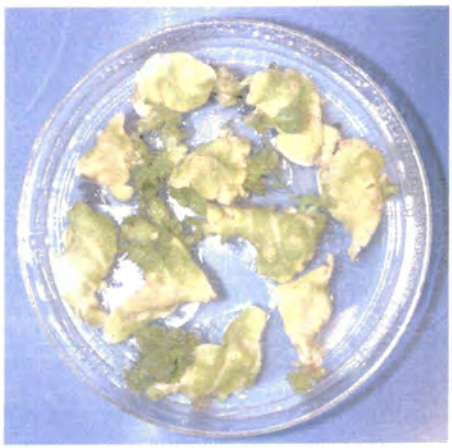
On the basis of the entire coding sequence of the vine weevil chitinase clone determined earlier, a pair of PCR primers were designed to the 5′ (5′-CCGGATCCATGGGCAAAAGGTTAATTC-3′), and 3′ (5′-CGAGCTCTTAGCAAACAACTGCGGAGATCCAGTTGCA-3′) ends of the mature polypeptide. The primers were designed to incorporate unique restriction sites to aid cloning in subsequent downstream operations. Once the coding sequence had been amplified with a proof-reading polymerase (Expand, Roche), the resulting product was separated by agarose gel electrophoresis, purified and TA cloned into pCR2.1 as usual, before being transformed into TOP10 electrocompetent *E.coli*. The plasmids from six recombinant bacteria were isolated and analysed for the presence of the original PCR fragment by restriction digestion with *EcoR*I, a single positive clones was chosen to be sequenced to ensure faithful duplication of the original cDNA clone. This plasmid was then restricted with *BamH*I (cuts once within the 5′ PCR primer) and *Sac*I (cuts once within the 3′ PCR primer) to release the ORF of the chitinase clone, the binary vector pBI121 (Clontech) was also restricted with *BamH*I and *Sac*I to accept the chitinase ORF in the correct orientation as a transcriptional fusion to the CamV35S constitutive promoter. After ligation of the chitinase insert to the backbone of pBI121, and transformation into DH5α, recombinant colonies were screened by colony PCR. Plasmid DNA from a positively transformed colony was isolated and then retransformed into electrocompetent *A.tumefaciens* (LBA4404) for use in *Agrobacteria* mediated plant transformation.

5.3.2 Tobacco Transformation with Vine Weevil Chitinase

Tobacco transformation was performed as described by Gallois and Marinho (1995) using 1cm^2 leaf section for the acceptor material. Once regeneration of the callus (Figure 5-3) had begun 30 shoots were selected and transplanted to rooting media in small screw top vials (Figure 5-3). After several weeks 20 plantlets, with well developed root systems, were planted out in John Innes Number 3 compost and grown to maturity in controlled environment growth rooms at 24°C with a 16 hour photoperiod (Figure 5-3).

5.3.3 Analysis of Transformants

Once the plants became established RNA was extracted from 100mg of leaf tissue to undergo RT-PCR analysis. After purifying total RNA from each plant line using tri-reagent (Sigma), 1μg was used as a template for RT-PCR. First strand synthesis was initiated using the anti-sense primer towards the active site consensus sequence (Chitinase A.site) and PCR amplification was achieved using the sense primer designed for the cloning regime (3'ChiExp). The reaction used untransformed tobacco RNA, no RNA and a vine weevil chitinase cDNA clone as controls. Figure 5-4 shows the resulting agarose gel after electrophoresis of the products, with no amplification of products seen in the control tobacco and the no template controls (Lanes A and B respectively), where as positive amplification was seen against the plasmid control. Also when the RT-PCR was performed on the experimental plants each reaction gave differing amounts of product (Figure 5-4), suggesting the transgene was being transcribed at different levels between each plant.



Regenerating shootlets
on shooting media

Root formation of
shootlet

Full regenerated plantlet
ready for potting on, into soil

Established regenerated
transgenic tobacco lines

Tobacco seeds ready
for harvesting

Figure 5-3 Pictures illustrating the regeneration of tobacco plants from tissue culture transformation to mature seed producing plants

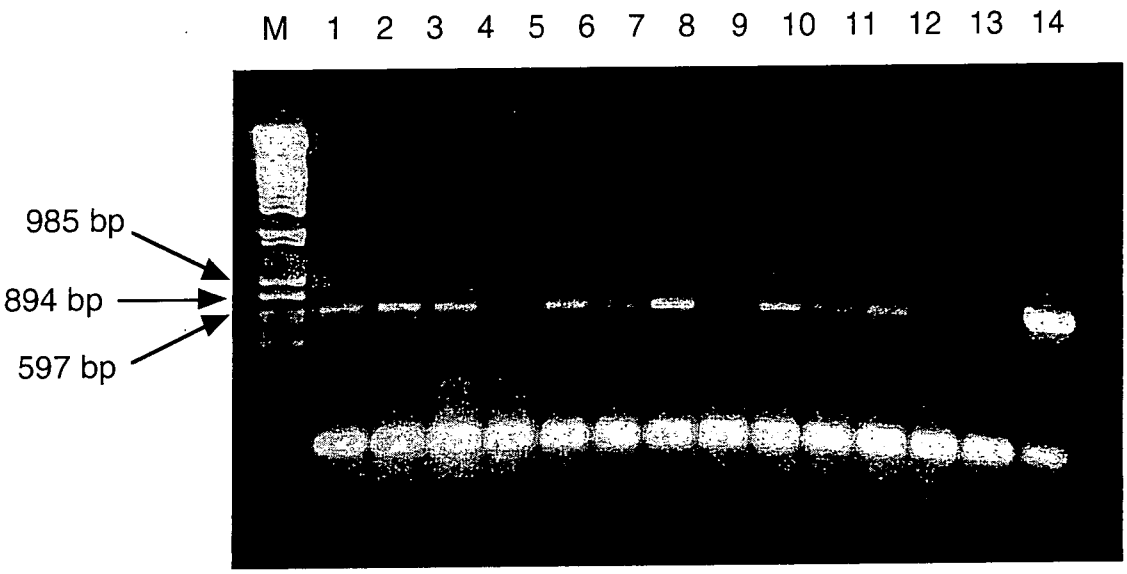


Figure 5-4 Agarose gel (1.5%) showing amplified products (600 base pairs) from the RT-PCR of primary transformed tobacco plants expressing the full length vine weevil chitinase cDNA clone. Lane M; Eco471 molecular weight markers, lanes 1-11 transgenic experimental plant lines VWChi 1 through VWChi 11 respectively (1μg total RNA used to initiate reaction), lanes 12, 13 and 14; control tobacco (1μg), no RNA control, vine weevil chitinase cDNA clone in pBluescript (positive control)

5.4 Discussion

Enzymes that specifically hydrolyse the linear homopolymer of 2-acetamide-2-deoxy-D-glucopyranoside (*N*-acetylglucosamine, GlcNAc) joined by ß-1,4 linkages (Chitin) are known as chitinases. Chitinases cleave at several internal sites throughout the polysaccharide chain resulting in the production of soluble low molecular mass multimers of GlcNAc. Of these products the smallest oligosaccharides predominate; chitobiose and chitotriose (Kramer and Reynolds). These short multimers of GlcNAc are subsequently processed by another chitinolytic enzyme, ß-N-acetylglucosaminidase which removes GlcNAc units sequentially from the non-reducing end of the sugar substrate (Fukamizo, 1985). Previously chitinases have been identified in the moulting fluids, venom glands and midguts of several insect species (Kramer, 1997). Their role is believed to be responsible for the shedding of old exoskeletons, particularly when proceeding to a new larval instar, and for the degradation of the peritrophic membrane of the gut which is either continuously or periodically re-synthesized (Lehane, 1996).

The identification of chitinases in the midgut of insects has been postulated to have some role in digestion above that of peritrophic membrane turnover. The work presented here shows the identification of such an enzyme whose cDNA was isolated from a gut specific library generated from vine weevil larvae.

The use of a 5'RACE ready library of gut cDNA as a template from which to generate a homologous probe to screen a gut specific cDNA lambda phage library was in this case advantageous as only one degenerate primer was needed for the initial amplification step. Generally a pair of degenerate PCR primers can be designed by the reverse translation of conserved regions of the protein under investigation. However previous alignments of chitinolytic enzymes showed that the two highly conserved regions which would have been ideal candidates for priming sites, were too close together to be used as sites for amplification (expected product size 150bp). So by choosing the conserved region containing the active site (FDG[L/F/V]DLDWEYP) an anti-sense degenerate primer was constructed, and by using this in tandem with the universal primer (Clontech) which was homologous to the 5'SMART oligonucleotide (Clontech) situated at the 5'end of the 5'RACE ready cDNA, the amplification could be performed under more stringent conditions than if two degenerate primers were used.

The identification of the resulting PCR product as a fragment of a chitinase allowed it to be used as a probe to screen the previously constructed lambda phage gut cDNA library. Screening resulted in the isolation of the remaining sequence which, when submitted to BlastP searching confirmed the initial findings suggesting the sequence to be that of a chitinase. The total theoretical cDNA clone is 1795 base pairs in length containing an ORF equivalent to 504 amino acids. The theoretical cDNA clone contains a 5′ UTR of some 110base pairs, immediately preceding the ATG start codon. A signal peptide cleavage site was located between residues 21 and 22, indicating the peptide is directed out of the cellular cytoplasm and through the cell wall to its final extracellular destination. The 3′ end of the ORF is bounded by a stop codon (TAA), and 135 bases downstream of this is a consensus polyadenylation signal, followed by a poly-A tail of some 20 consecutive adenine bases. The resulting protein product has a predicted molecular weight of 53.6KDa and a pI of 4.16. Pair-wise alignments of the vine weevil chitinase with those of; *A.gambiae*, *Chelonus sp.*, *P.cochleariae* show a high degree of conservation throughout the catalytic domain (Figure 5-2) including the area surrounding the proposed active site motif which contains the active tryptophan identified by Zhang *et al* (2003) as shown in Figure 5-2. The multi-domain architecture of known chitinases is again mirrored with the vine weevil clone (Arakane *et al*, 2003), previous multiple alignments have shown a conserved structural motif at the C-terminal end of chitinase sequences containing six cysteine residues, Figure 5-2 highlights this motif and shows its presence in the vine weevil clone. Tellam (1996) first discovered that this motif is shared between several peritrophic matrix proteins, and earlier with a range of receptors and other proteins controlling cellular adhesion (Tellam *et al* 1992). The final domain found in a majority of chitinases is a region rich in proline, glutamate, serine and threonine residues (PEST domain), which precedes the cysteine rich carboxy terminus. The PEST region has been implemented to increase the susceptibility to proteolysis by calcium-dependent proteases (Rogers *et al* 1986, Arakane *et al*, 2003). Sequence analysis of the vine weevil chitinase clone showed that this PEST domain had been replaced by another region, which was rich in serine, aspartate and threonine (SDT) residues. This domain shows very high levels of organisation with six consensus repeats of SSSSDT and a single ASSSDT sequence. This SDT domain has not been observed previously in chitinases and differs from the classical PEST domain, and the alternate sequences seen in *Serriata* and chitinases isolated from prawns.

The cloning of this chitinase from the vine weevil gut library has shown an adaptation of the classical multi-domain architecture of these enzymes, with the replacement of the

PEST region with repeats of serines, aspartates and threonines. The isolation of this clone serves as a starting point for the production of transgenic plants expressing the vine weevil chitinase enzyme as a biopesticide (Kramer, 1997).

5.4.1 Expression of the Vine Weevil Chitinase in Tobacco for use as a Biopesticide

The ability to generate plants expressing novel and foreign genes with a potential biopesticide effect is increasingly becoming an important factor in the protection of economically important crops. Since the first reports of this technology in 1984 (Horch *et al* 1984) we have continued to develop and harness its ability, with the majority of research focussing upon its use in enhancing crop protection, and thus reducing our current indiscriminate reliance upon chemical based insecticides and fungicides.

This work shows how the cDNA encoding a chitinolytic enzyme isolated from the gut of the vine weevil has been stably transformed into tobacco plants. Analysis of the regenerated plants, which survived antibiotic selection in tissue culture, by RT-PCR showed that 81.8% (9/11) of the population were transcribing the chitinase mRNA at a verity of concentrations per 1μg of total RNA used to prime the RT-PCR reaction (Figure 5-4), with no non-specific amplification of endogenous tobacco chitinases. The introduction of this insect cDNA and the subsequent expression of the encoded protein in the tobacco did, on the whole, not cause any obvious phenotypic changes as compared with those of wild type un-transformed parental tobacco, except in one case. This line (VWChi 7) did not develop into a normal plant within the time scale of the other transformed lines, its leaves did not expand as would have been expected (only reaching 30 x 50mm), the epidermis appearing to be thickened with a crusty/crystalline texture, the overall height of the plant was reduced and the plant did not reach sexual maturity, so no seeds could be collected. Interestingly the RT-PCR analysis of VWChi7 showed that it had the greatest levels of transcribed message (Figure 5-4). Whether these phenotypic abnormalities were a result of a toxic effect originating from the expression of the vine weevil chitinase or due to regeneration in tissue culture cannot be determined.

Previous studies involving transgenic tobacco expressing a tobacco hornworm (*Manduca sexta*) chitinase (Ding *et al* 1998) show the T_1 plants express a truncated, but enzymatically active chitinase. Throughout bioassays the transgenic lines showed

significant levels of resistance to the tobacco budworm (*Heliothis virescens*), but none against the tobacco hornworm. Analysis of the active truncated chitinase expressed in the transgenic line predicted cleavage at the C-terminal end of the mature peptide (Wang *et al* 1996), presumable within the PEST region which has been proposed as a target for calcium dependent proteases and facilitates protein turnover (Rogers *et al* 1986). The results of this proteolytic cleavage are a reduction of the specific activity of the expressed protein ($^1/_4$ of that of the full length enzyme, Wang *et al* 1996). Protein analysis and subsequent bioassays will show if the vine weevil chitinase, with its unique SDT region is more stable than the traditional PEST region and will yield greater quantities of full length active protein.

The use of insect chitinases alone as biopesticides suggests that they have a greater effect on alternate species within an order, than they do on the original species from which the cDNA was derived. Whether this will be the case across different orders further work will show (Ding *et al* 1998). Furthermore Ding *et al* (1998) showed that the presence of a chitinase transgene enhances the effects of a non-lethal dose of *B.thuringiensis* toxin (288mg/g fresh leaf weight, by folia application) towards both budworm and hornworm. The effectiveness of chitinases as biopesticides is surely one which we should take seriously, especially if the transgene can be engineered to be more stable by the fusion of the active catalytic domain to other gut binding moieties, such as lectins. Additionally the synergistic effects seen with the Bt toxin could lead to intelligent crop spraying protocols or the use of gene pyramiding in transgenic plants (Datta *et al*, 2002) with other resistance factors could increase the efficacy of each transgene giving greater resistance with the aim to reducing the occurrence of Bt resistant insect cultivars.

6 Vine Weevil Gut Cellulase

As previously described in Chapter 3 random screening of cDNA clones from a gut specific cDNA library generated from larval vine weevils identified a sequence showing strong similarity to a cellulolytic enzyme. Further homology analysis determined the enzyme to be a member of the glycosyl hydrolase family 48, which are secreted exocellulases known as cellobiohydrolases (CBH).

The original clone extracted from the library was found to be truncated at its 5' end, but did contain a predicted ORF. This ORF was bounded at the 3' end of the sequence by a stop codon at position 1911. This stop codon is followed by a putative 50bp 3'UTR containing a single consensus polyadenylation signal and a poly-A tail of 24 consecutive adenine residues.

6.1 Identification of the Terminal 5' Region

The technique of 5'RACE (Rapid Amplification of cDNA Ends, Clontech) was used to amplify the 5' region of the cellobiohydrolase mRNA, using total RNA isolated from vine weevil gut tissue as a template. The anti-sense primer was designed to a region 550-569 bases downstream of the first known reliable sequence. This primer was then used in tandem with the universal primers supplied by the manufacturers, in an RT-PCR reaction. A fragment of 670bp was amplified using the supplied proofreading polymerase. The resulting PCR product was cloned, analysed and sequenced in the usual fashion, and sequence analysis showed the product to be identical to the previously determined region of the original sequence up to and including the anti-sense priming site at the 3' end of the 5'RACE PCR product, except for the addition of 62 extra bases at the 5' end of the cDNA. The 5'RACE sequence located the previously unidentified ATG start codon, giving a complete ORF of 1912 bases. The sequence data also showed a region of 60 bases upstream of the start codon containing two stop codons at –40 and –45 bases. Figure 6.1 shows the strategy used in sequencing clone VW24 and figure 6.2 shows the completed vine weevil cellobiohydrolase cDNA sequence.

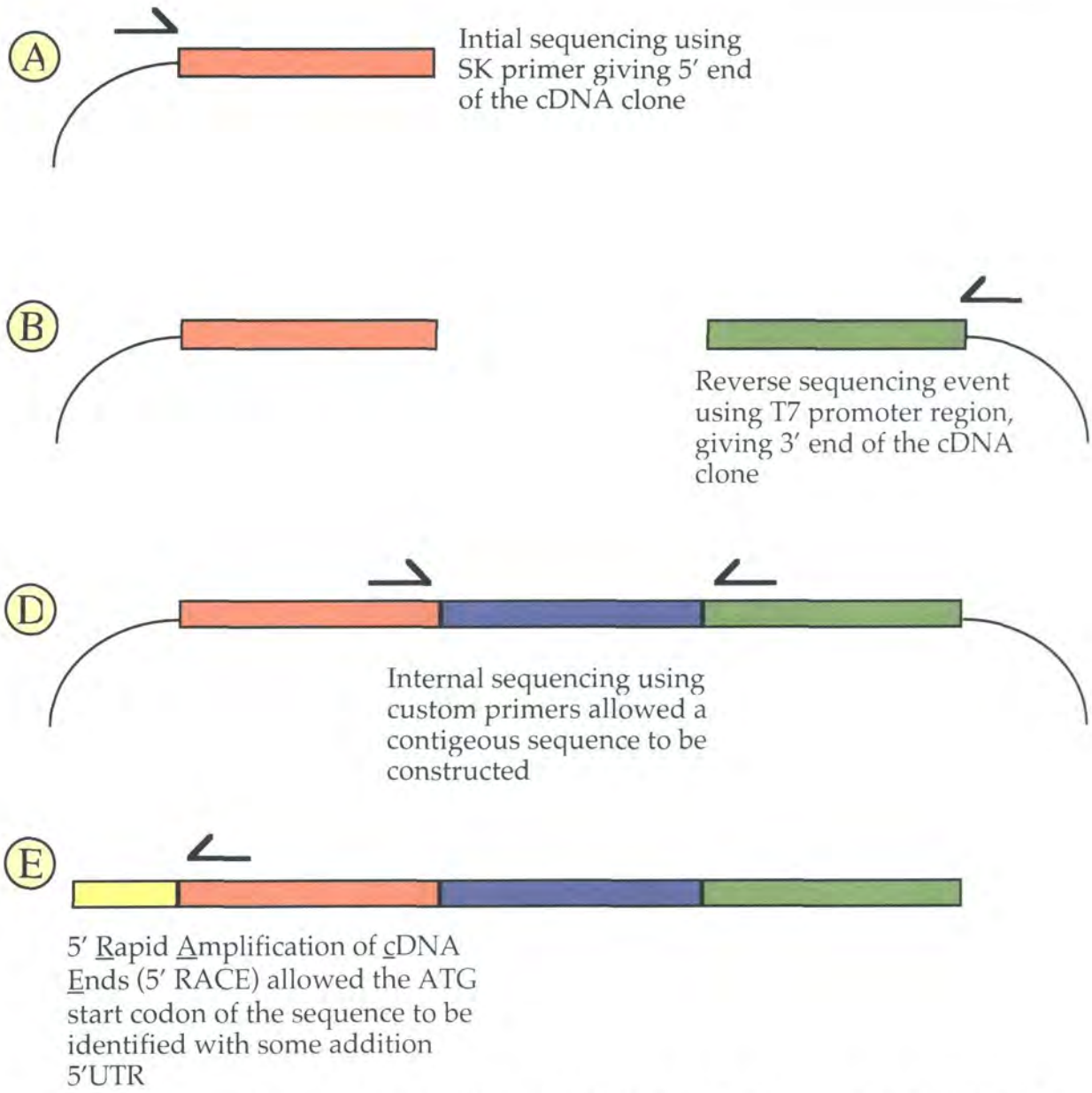


Figure 6-1 Sequencing strategy used to allow identification of the complete vine weevil cDNA sequence.

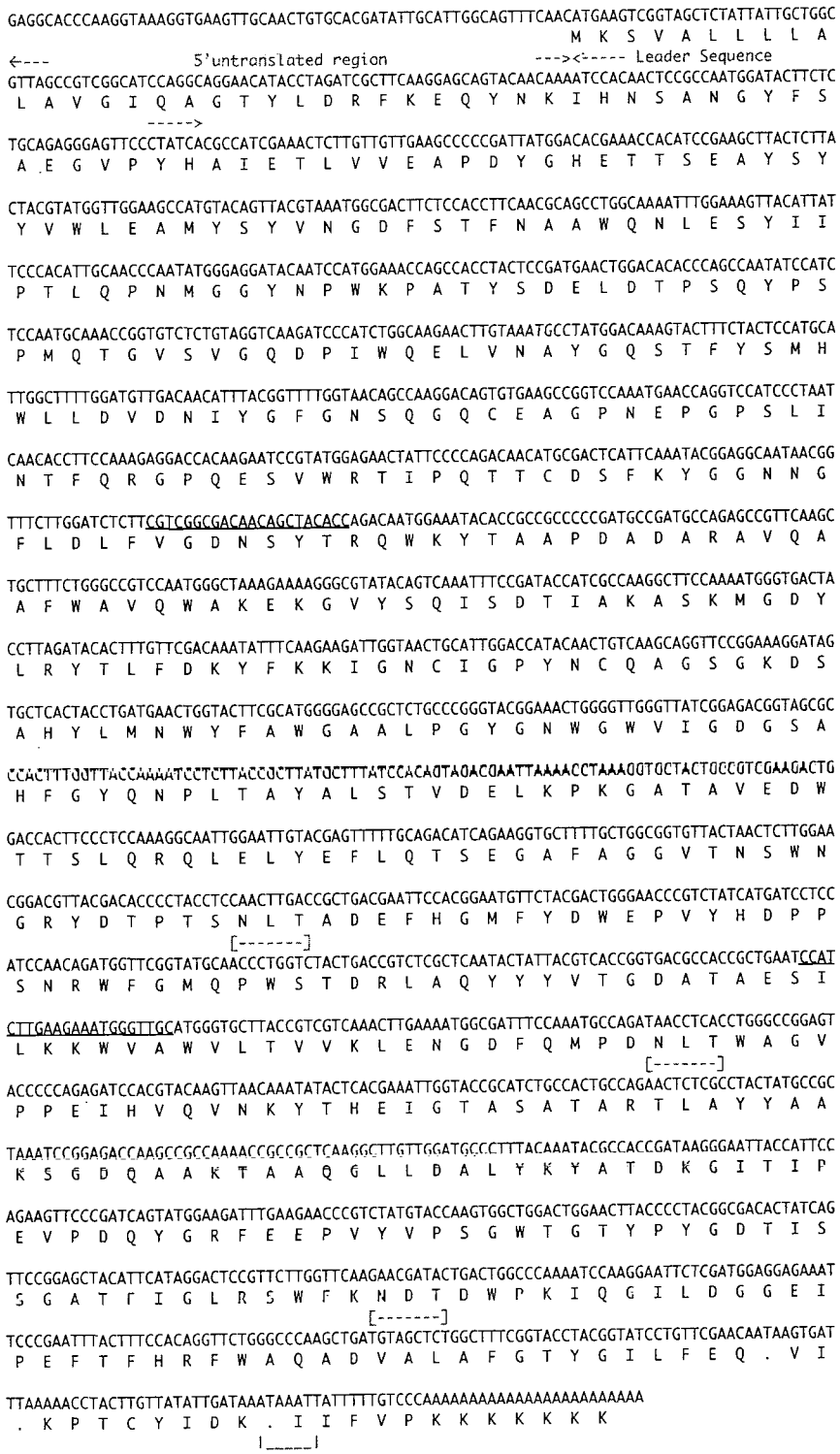


Figure 6-2 The completed vine weevil cellobiohydrolase (VW24) cDNA clone with 5'URT amplified through 5'RACE using the priming region shown (...), the sequence is also annotated to show the predicted translational product, putative N-glycosylation sites ([---]), consensus poly adenylation signal (|__|), the signal peptide sequence (<-->) and the internal pair of primers used to complete the full open reading frame (...).

6.2 Cellulase Activity within the Vine Weevil Larval Gut

The isolation of a cDNA clone encoding cellobiohydrolase implied that the corresponding enzyme activity, and by inference a complete cellulose digestion system was present in the vine weevil larvae. Assays were therefore conducted to confirm the capacity of this insect to digest cellulose.

6.2.1 Gut Cellobiohydrolase Activity

Total soluble proteins were extracted from 5 larval vine weevil guts by homogenisation in 100μl dH_2O, after which any non-soluble residue was removed by centrifugation. 5μl of a 1/10 dilution of the crude gut extract was used to analyse *in vitro* CBH activity. Hydrolysis of the substrate 4-methylumbelliferyl-ß-D-cellobiose was performed over a pH range of 3.5 to 10, following incubation at 37°C for three hours each reaction was quenched with 1M NaOH to ensure maximal fluorescence of the 4-methylumbelliferol before reading on a fluorescence microtitre plate reader. Figure 6- shows the resulting mean rate of hydrolysis against pH . The data shows a peak of cellobiohydrolase activity between pH 4 and 6, with maximal activity occurring at pH 4.5-5.0.

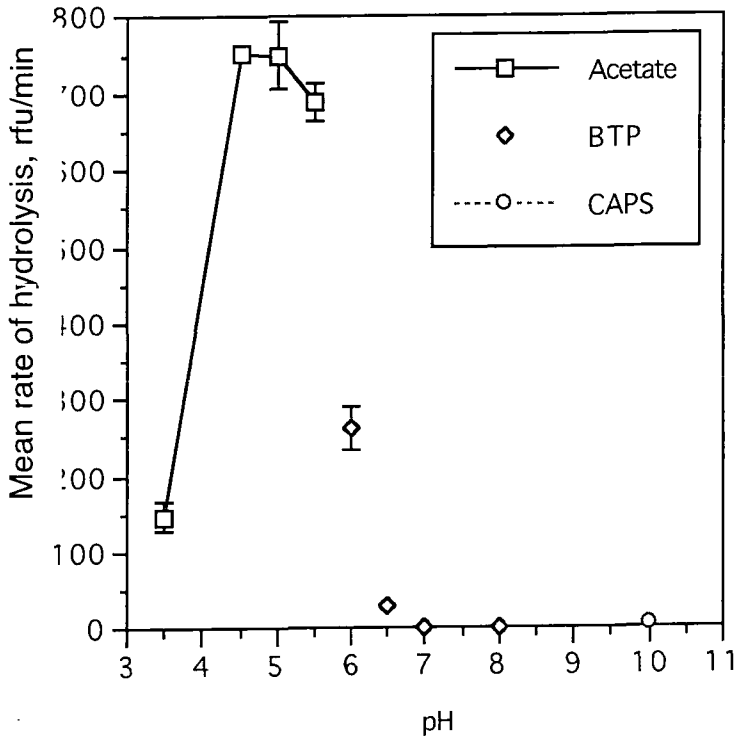


Figure 6-3 Cellobiohydrolase activity within crude gut extracts of vine weevil larvae. Assay performed over a pH range of 3.5 to 10 against the fluorescent substrate 4-methylumbelliferyl-ß-D-cellobioside. Enzymatic reactions were performed for 3 hours at 30°C, and quenched with 1M NaOH before reading to maximise fluorescence. Points and bars represent the mean ± the standard deviation across triplicate samples.

6.2.2 Cellulose Digestion

The previous experiment describes how CBH activity was demonstrated in crude extracts of vine weevil larval guts. However several cellulolytic enzymes are believed to act synergistically upon insoluble cellulase to release soluble glucose units (Figure 6-3). To determine whether such enzymes were present in the vine weevil a crude extract of the vine weevil larval gut was used in a total cellulolytic degradation cascade as shown in Figure 6-4 (Amplex Red, Molecular Probes).

Cellulose digestion was assayed as suggested by the manufacturers of the Amplex Red kit, using the control *T.reesei* cellulase to generate a standard curve of cellulase activity. Control cellulase was added at the equivalents of 0.4, 4, 10 and 40 mU/ml. Figure 6-5 shows the results gained from the assay performed using 2, 0.2 and 0.02μl of a 1:10 dilution of crude vine weevil gut extract, equivalent to 0.02, 0.002 and 0.0002 vine weevil larval guts. The ΔRFU for this serial dilution of the crude gut extract do not reflect the linear nature of the dilution, this may be due to the limitations of the detection method or errors incurred during the experimental procedure.

The assay was run with and without the inclusion of 200mU ß-glucosidase. The graph shows that total cellulose digestion has occurred in the gut samples where no additional ß-glucosidase was included, with the 2μl sample giving a relative change in fluorescence of 8000 units, the 0.2μl a ΔRFU of 7.12 units (not shown in graph). However the data shows that when ß-glucosidase is added to the reaction the ΔRFU increases for each sample allowing celluloytic digestion at the lowest concentration of gut extract, 0.02μl gut extract plus ß-glucosidase giving a ΔRFU of 1000 units.

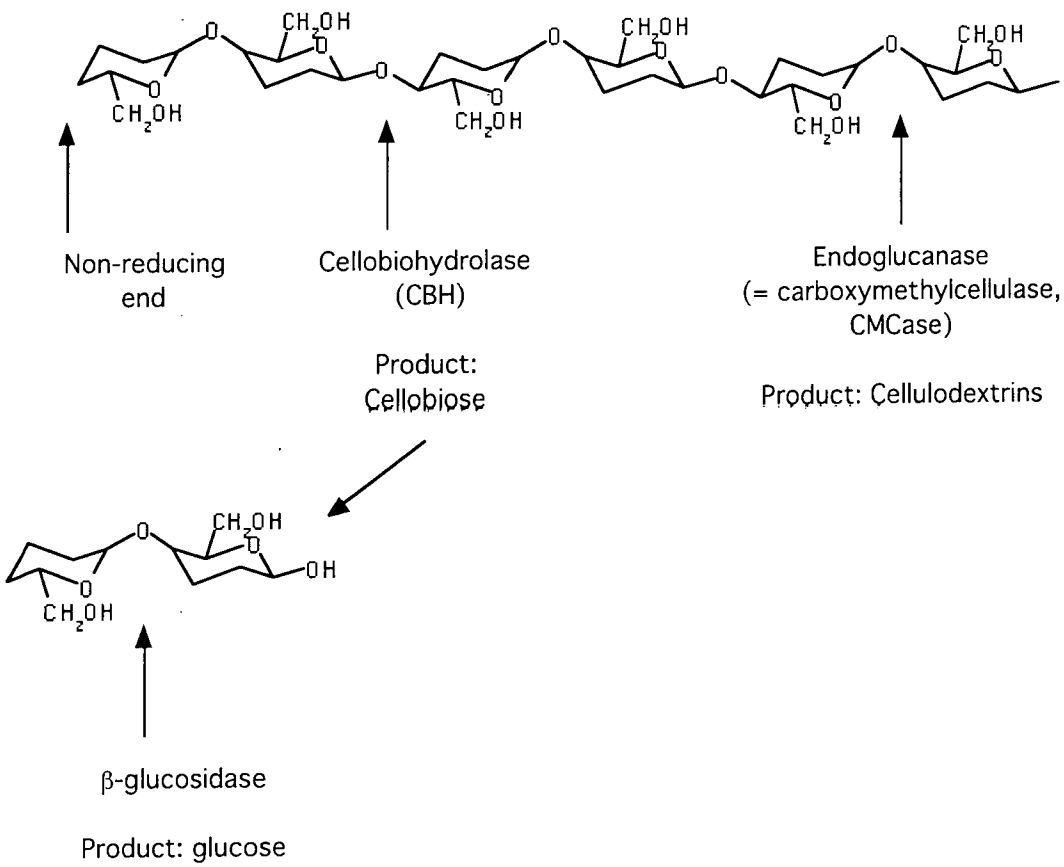


Figure 6-3 Overview of the digestion of crystaline cellulose. CBH attacks microcrystalline cellulose; hydrolyses glycosidic bond 2 in from non-reducing end; usually rate limiting in cellulose degradation. CMCase attacks randomly within cellulose. All three enzymes act synergistically to degrade cellulose

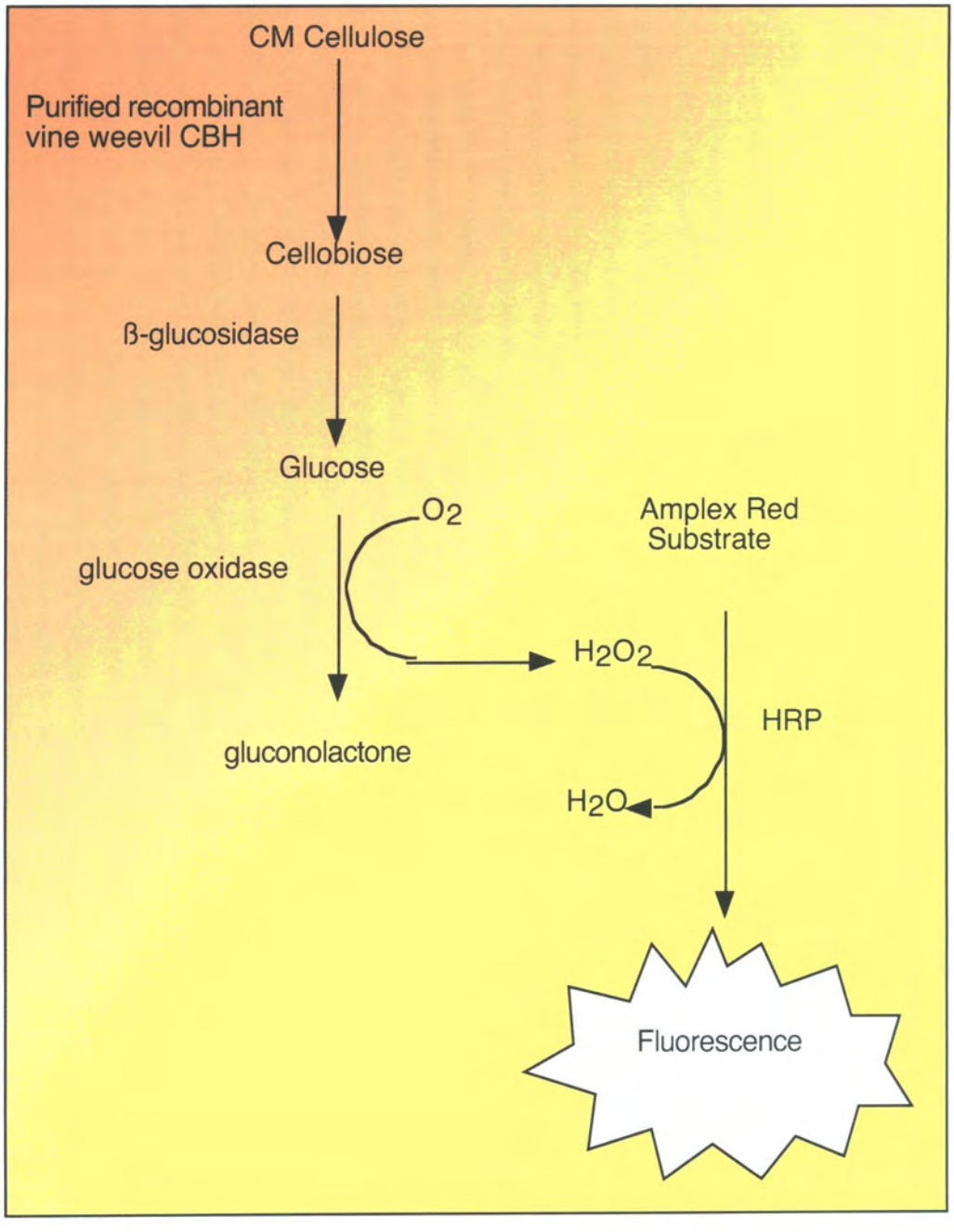


Figure 6-4 Flow diagram showing the enzymatic reactions involved in the Amplex Red (Molecular Probes) total cellulose digestion detection kit. Fluorescence was measured in an Ascent Fluorimeter.

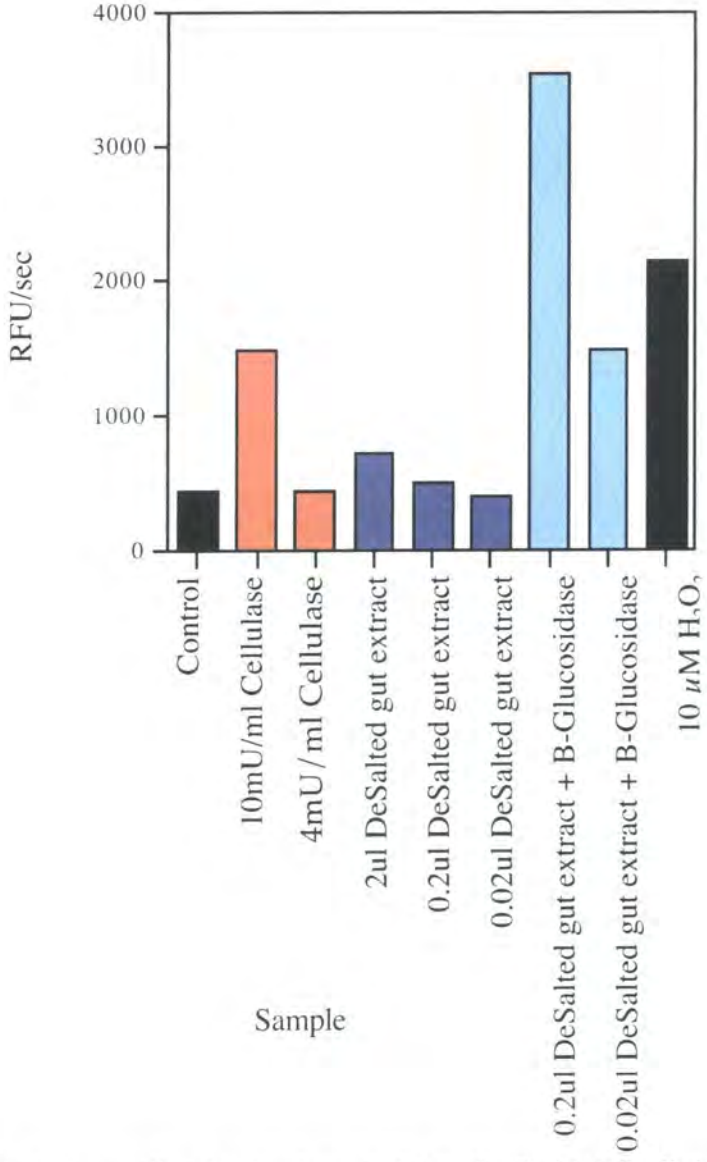


Figure 6-5 DeSalted vine weevil gut extract against AmplexRed total cellulase substrate, with and without the addition of exogenous ß-glucosidase. Lane 1; no enzyme control, lanes 2-3; *T.reesei* cellulase control with ß-glucosidase, lanes 4-6; vine weevil crude gut extract without ß-glucosidase, lanes 7-8; vine weevil crude gut extract with ß-glucosidase, lane 9; 10μM H_2O_2 redox reaction control.

6.2.3 Gut ß-Glucosidase Activity

To confirm the vine weevil contained the inherent ability for complete cellulose digestion and not just simply cellobiohydrolase activity as predicted by the molecular analysis of the cDNA clones, it was decided to look for the activity of ß-glucosidase. ß-glucosidases are typically known to be the final enzyme of the cellulose digestion cascade.

The ß-glucosidase activity in crude extracts of vine weevil larval guts was measured using the fluorimetric substrate 4-methylumbelliferyl-ß-D-glucoside (Molecular Probes). Figure 6-6 shows the change in relative fluorescence units between 42 and 60 minutes for successive 10 fold dilutions of the crude gut extract. The graph shows that when increasing amounts of gut extract are added, hydrolysis of the substrate increases to levels above those seen with the controls.

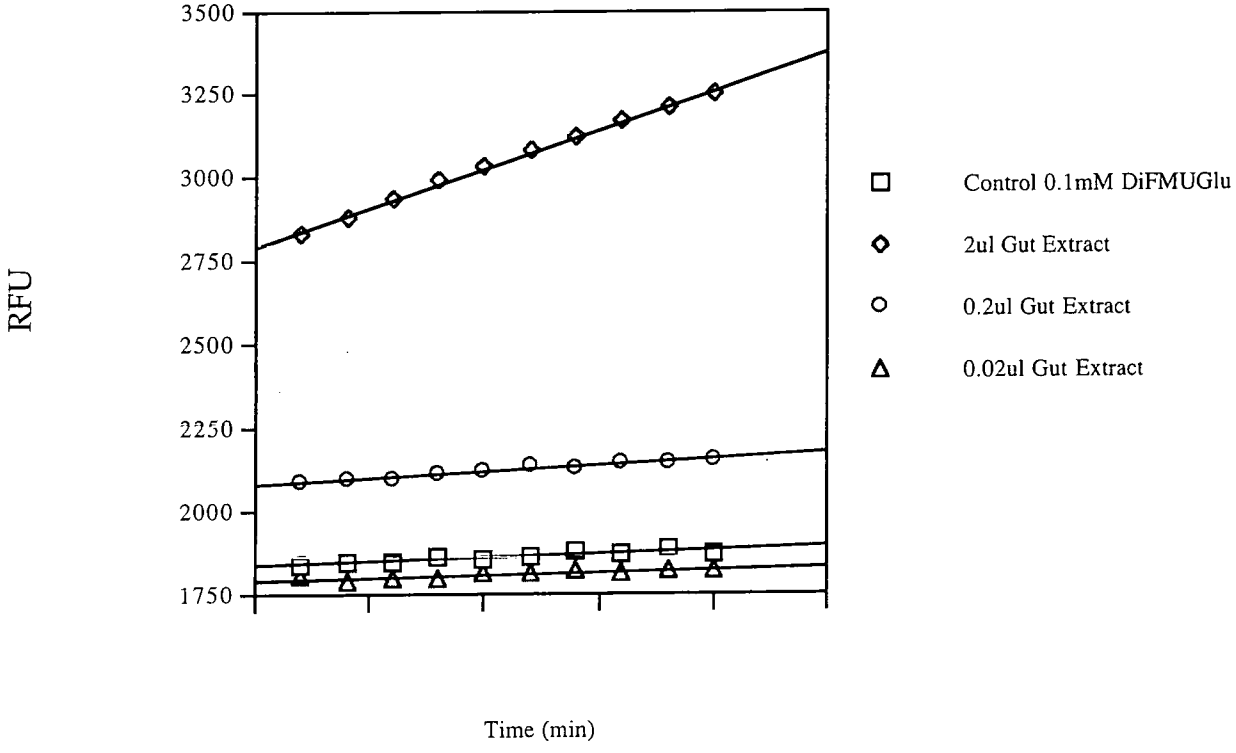


Figure 6-6 ß-Glucosidase activity within crude extracts of vine weevil larval guts. Assays performed against DiFMUGlu (Molecular Probes) fluorescent substrate and incubated at 42-60 min in 0.1M Acetate buffer (pH 5.0)

6.3 Cloning and Expression of Vine Weevil Cellobiohydrolase

To examine whether the cellobiohydrolase clone isolated from the cDNA library had the ability to encode a functional enzyme, and therefore could be responsible for the cellobiohydrolase activity seen in vine weevil gut extracts it was necessary to express the cDNA clone in a heterologous expression system.

6.3.1 Bacterial expression and purification of recombinant protein

The mature coding sequence from the clone VW24 was amplified by PCR using primers that contained unique restriction sites at each end of the sequence. The PCR product was isolated after separation by gel electrophoresis and cloned into pCR2.1 as an intermediate vector using the T-A cloning method. The insert was excised from a verified clone in pCR2.1 by restriction digestion, purification and ligated to the expression vector pET24a which had been similarly restricted with NdeI and XhoI. The ligation mixture was transformed into *E.coli* strain BL21DE3. After selection of a clone containing the recombinant plasmid by plating on selective media and mini-preparation of plasmid DNA, a verified clone of the expression construct was used to express the recombinant protein. Figure 6-7 shows an SDS-PAGE gel of the insoluble and soluble fractions from bacterial cultures that had been induced with IPTG, and those that had not been induced. The gel shows the presence of an additional protein band in the insoluble protein fraction from induced bacteria, at the predicted molecular weight for the recombinant vine weevil cellobiohydrolase (~66 KDa), as estimated from the sequence analysis. This analysis suggested that the cDNA sequence was being expressed at a level sufficient to allow protein isolation.

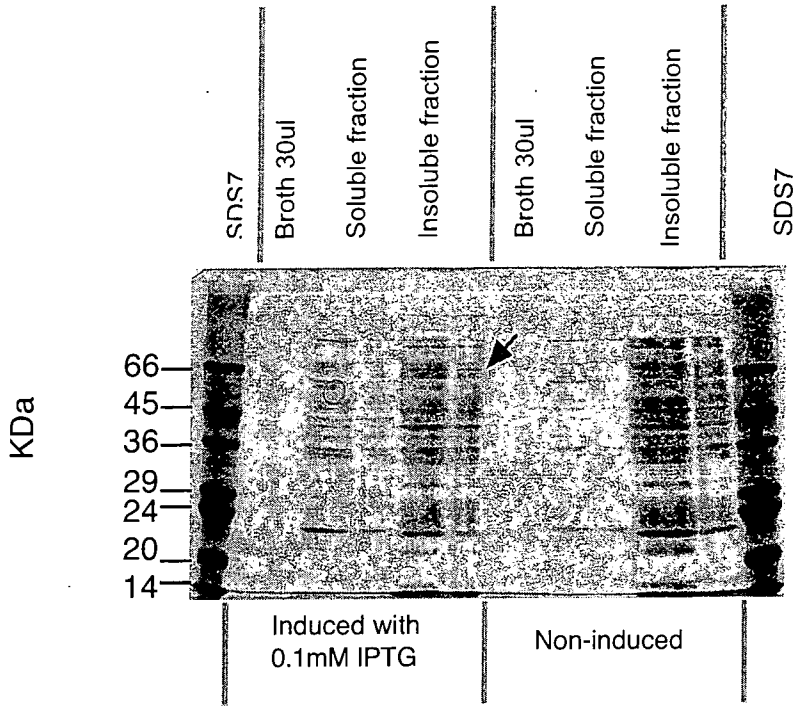


Figure 6-7 SDS-PAGE gel showing proteins extracted from the soluble and insoluble fractions of induced and non-induced E.coli (BL21DE3) containing the recombinant Cellobiohydrolase clone (VW24). The arrow indicates the presence of a band in the induced fraction that is of the correct molecular weight for the recombinant protein.

The PCR cloning primer used at the 3′ end of the construct contained a sequential run of six histidine residues (6x His tag) prior to the stop codon. Once translated, this sequence gives a C-terminal His tag which allows purification of the rCBH on a nickel affinity column. The insoluble protein fraction from a culture of the expression clone was prepared, solubilised in denaturing solvent, and purified by nickel affinity chromatography under denaturing conditions. Figure 6-8 shows an analysis of the eluted peak fractions from the nickel affinity column by SDS-PAGE, along with the original load fraction. The SDS-PAGE gel shows that the recombinant protein has been purified from endogenous *E.coli* proteins by affinity chromatography. The protein was purified to a high degree, although there still remained some additional lower molecular weight proteins present on the gel. These may be products of proteolytic cleavage of the recombinant CBH that still retained the His tag.

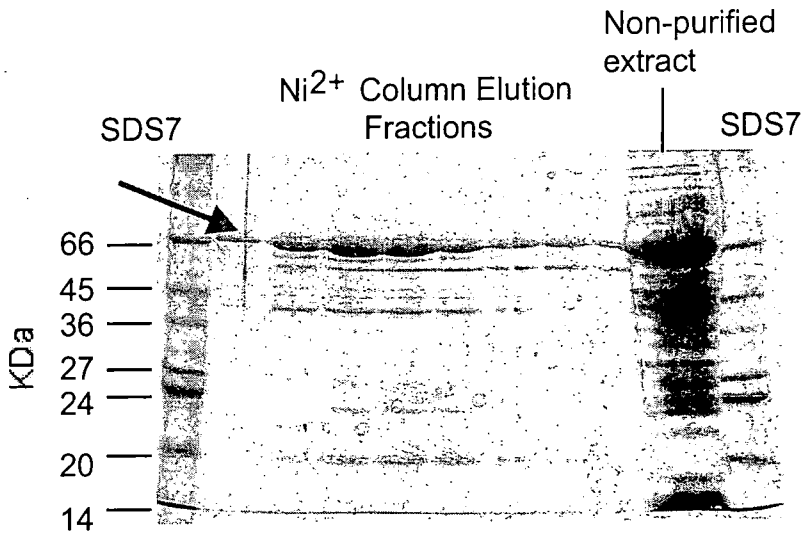


Figure 6-8 SDS-PAGE gel showing the eluted peaks collected from the purification of the bacterially expressed recombinant vine weevil cellobiohydrolase (clone VW24) after elution from a Ni^{2+} affinity column. Total insoluble extract was; loaded in 20mM Tris-HC pH 8.0, 0.5M NaCl, 5mM Imidazole, 8M Urea; washed in 20mM Tris-HC pH 8.0, 0.5M NaCl, 20mM Imidazole, 8M Urea; and eluted in 20mM Tris-HC pH 8.0, 0.5M NaCl, 300mM Imidazole, 8M Urea. The arrow indicates the eluted rCBH protein.

6.3.1.1 Activity of recombinant CBH expressed in E.coli

The expressed protein was largely insoluble so 8M urea was used for extraction before recovering the protein on the Ni^{2+} affinity column. The eluted peak fractions from the nickel affinity column were pooled together, and dialysed overnight against 0.1M Acetate buffer pH 5.5 to refold, and to re-activate the enzyme. Aliquots of the dialysed enzyme were introduced into the 4-methylumbelliferyl-ß-D-cellobiose assay as used earlier, however no activity was seen. This process was repeated several times with alterations to the parameters of dialysis, but no activity was recovered from expressing the vine weevil cellobiohydrolase clone in this type of expression system.

6.3.2 Expression in Pichia pastoris

The vector used to express the vine weevil cellobiohydrolase in this eukaryotic expression system contains a signal peptide which directs the recombinant protein for extracellular deposition into the culture supernatant in an active form (pGAPzα, Invitrogen). The system allows large amounts of protein to be expressed and then isolated from the culture supernatant without the need for the strong denaturants needed in the previous bacterial expression system used. Cloning of the recombinant cellobiohydrolase is performed in a two stage process, firstly the cDNA of the recombinant protein is cloning into a bacterial vector, this circular vector is then cut once with a single restriction enzyme to give a linear molecule. This DNA is then transformed into competent yeast where it is inserted into the yeast genome through the process of homologous recombination.

The mature coding sequence of VW24 was amplified by PCR (Pit5'Cell and Pit3'Cell) to incorporate two unique restriction sites at the 5' and 3' ends of the transcript. The PCR product was isolated by excising the appropriate band from an analysis of the reaction by agarose gel electrophoresis and was cloned into the intermediate vector pCR2.1 by the T-A cloning method. Plasmid DNA from a verified clone of this intermediate construct was digested with XhoI and XbaI and ligated to the *Pichia* expression vector pGAPzα which had also been restricted with the same enzymes. The expression construct was cloned in *E.coli* strain ToP10. Recombinant clones were selected by plating followed by plasmid mini-preps, and DNA sequencing of selected clones to check the construct. The expression constructs generated a transcriptional fusion with the α-factor sequence of the yeast expression vector pGAPzα (Invitrogen). Plasmid DNA from the construct was prepared from a culture of a verified clone, linearised by restriction with BlnI, and used to transform cells of *Pichia pastoris*. Transformed yeast containing integrated expression constructs were selected by plating on zeocin-containing media. Recombinant colonies of *P.pastoris* were grown in liquid culture, at 30°C. After 96 hours culture medium was collected and proteins were precipitated with ammonium sulphate. The proteins in the culture medium were analysed by western blotting using anti-His antibodies. The results of this analysis was not conclusive as recombinant protein could not be identified using this method, even though colonies were identified as containing the cloned CBH insert through colony PCR (data not shown).

6.3.2.1 *Purification and activity of Pichia expressed recombinant CBH*

Although the *Pichia* clones containing the cellobiohydrolase expression construct did not produce detectable cellobiohydrolase by Western blotting using antibodies against the His tag sequence, cellobiohydrolase activity was detected in the culture supernatant.

Figure 6-9 shows the activity of cellobiohydrolase in ammonium sulphate precipitated culture supernatant against 4-methylumbelliferyl-ß-D-cellobiose after purification by reversed phase chromatography. The assay was performed with the load, the flow through and the pooled eluted fractions from the column with a no enzyme control. The graph shows hydrolysis of the 4-methylumbelliferyl-ß-D-cellobiose substrate by the load fraction and that this activity is recovered in the pooled eluted fraction. No cellobiohydrolase activity was seen in the flow through or the wash fraction, likewise in the no enzyme control.

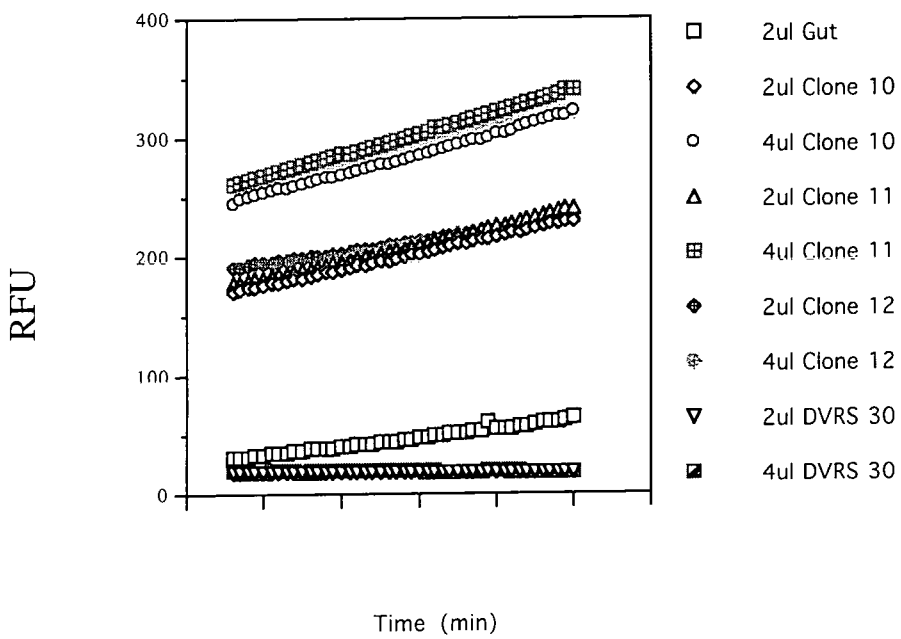


Figure 6-9 Rate assays of recombinant vine weevil cellobiohydrolase expressed in *P.pastoris*. Graph shows the hydrolysis of the fluorescent substrate 4-methylumbelliferyl-ß-D-cellobiose by ammonium sulphate precipitated culture supernatant take from the recombinant yeast after 72 hours of growth at 30°C. The assay was performed with 2µl vine weevil larval gut extract, and similar volumes of ammonium sulphate precipitated culture supernatant from yeast expressing a protease inhibitor (DVRS 30, pers comms) as suitable controls.

Reversed phase chromatography was repeated, this time with the eluate being collected in 2ml fractions. Figure 6-10 shows the retention time of the rCBH on phenyl sepharose at a flow of 1ml/min at room temperature against the ability of the eluent to hydrolyse the 4-methylumbelliferyl-ß-D-cellobiose substrate.

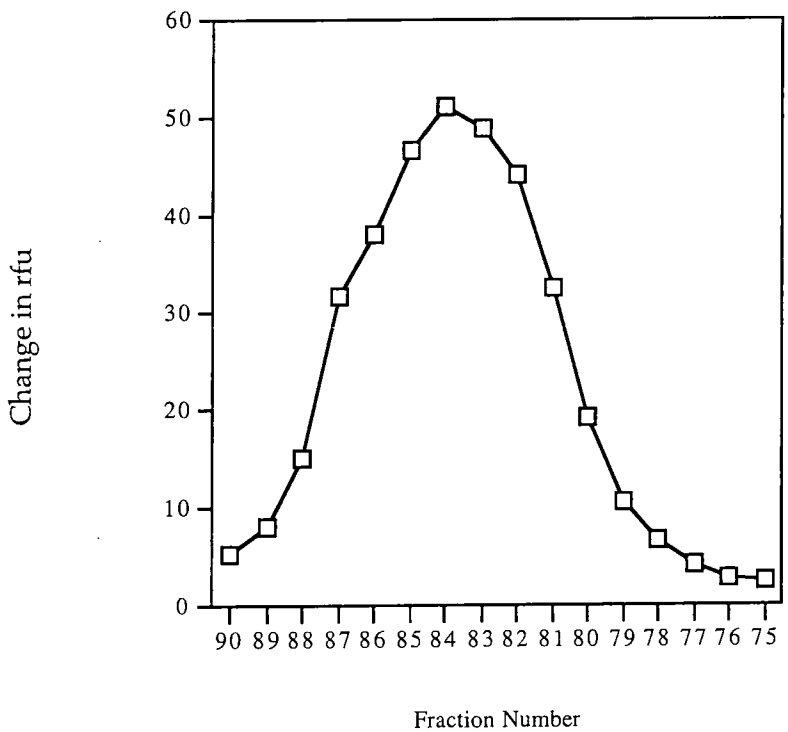


Figure 6-10 Change in RFU for reverse phase column fractions 75-90 (30-60 min retention time) of recombinant cellobiohydrolase expressed in *P.pastoris* against the fluorescent substrate 4-methylumbeliferylcellobiose in 0.1M acetate buffer (pH 5.0). Ammonium sulphate precipitated culture supernatant load in 4M NaCl and eluted in a linear gradient to 20mM Tris-Cl pH 7.8, and collected in 1 ml fractions.

6.3.2.2 *Recombinant cellobiohydrolase 'in gel' activity*

Ammonium sulphate precipitated fractions 75-90 were pooled and analysed using SDS-PAGE electrophoresis through gels co-polymerised with 0.1% CMC. Figure 6-11 shows the resulting congo red stained gel after incubation in activation buffer, the figure shows areas of clearing (unstained regions) where the CMC has been hydrolysed by the recombinant enzyme. These results are similar to those obtained with control cellulase (*Trichoderma reesei*, data not shown).

These experiments show that the cloned cellobiohydrolase encodes an active cellobiohydrolase that hydrolyses both 4-methylumbelliferyl-ß-Dcellobioside and cellulose.

Figure 5.11 show multiple bands with cellulolytic activity this is explained by the presence of lower molecular weight break-down products of the full length enzyme that still retain some catalytic activity. As seen in the final lane (lane 4) where the lowest concentration of column eluent was loaded the major band of activity can be contributed to the full length enzyme.

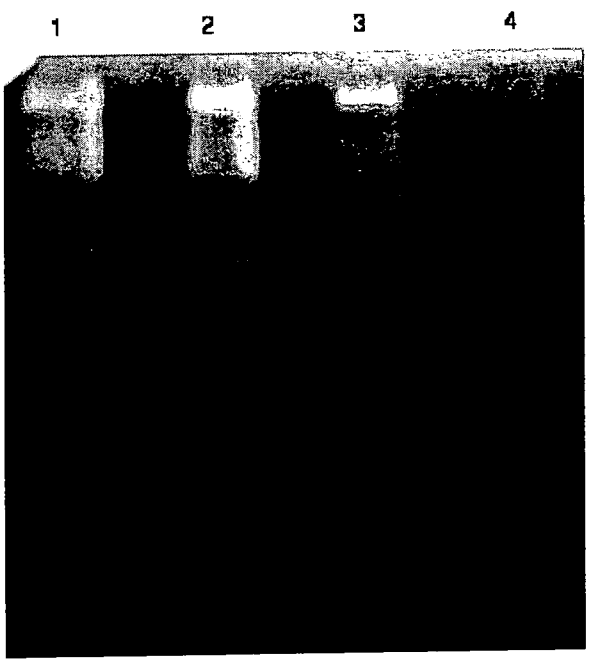


Figure 6-11 In-gel cellulase assay using pooled fractions of recombinant cellobiohydrolase after reverse phase chromatography. Regions of clearing indicate where the 0.1% CMC substrate has been hydrolysed by the activity of CBH eluted from the column. Lanes 1-4 contain 20µl, 15µl, 10µl, and 1µl column eluate respectively.

6.4 Genetic Origin of the Isolated Cellobiohydrolase cDNA

Cellobiohydrolases of the type represented by the cellobiohydrolase cDNA clone from the vine weevil are unknown in eukaryotes.

This section describes how the genetic origin of the isolated clone was investigated.

6.4.1 Vine Weevil Genomic Southern Blot Analysis

Figure 6-12 shows the resulting autoradiograph of a Southern Blot performed on 1μg of total genomic DNA extracted from the heads and bodies of vine weevil larvae. The Southern blot was probed with a ^{32}P-dCTP labelled Hinc*II* restriction fragment isolated from the vine weevil cellobiohydrolase cDNA clone. The digestion released a 999bp fragment which was also used as the positive control. The genomic DNA was digested with; Hinc*II* predicted to release the 999bp probe fragment; Hind*III* would release a fragment greater than 1.8kbp; Hind*III*/EcoR*I* was expected to release a fragment of approx 1kbp, and BamH*I* which was known not to cut within the cDNA and thus would give a predicted restriction fragment length of greater than 2.4kbp. As Figure 6-12 shows the restriction fragment lengths shown by the Southern blot are different from the predicted profile, but identical between the two different tissue types. Table 6.1 summarises the restriction fragment profile seen on the Southern blot showing both the distance migrated by the band through the agarose gel from the well and its relative molecular weight.

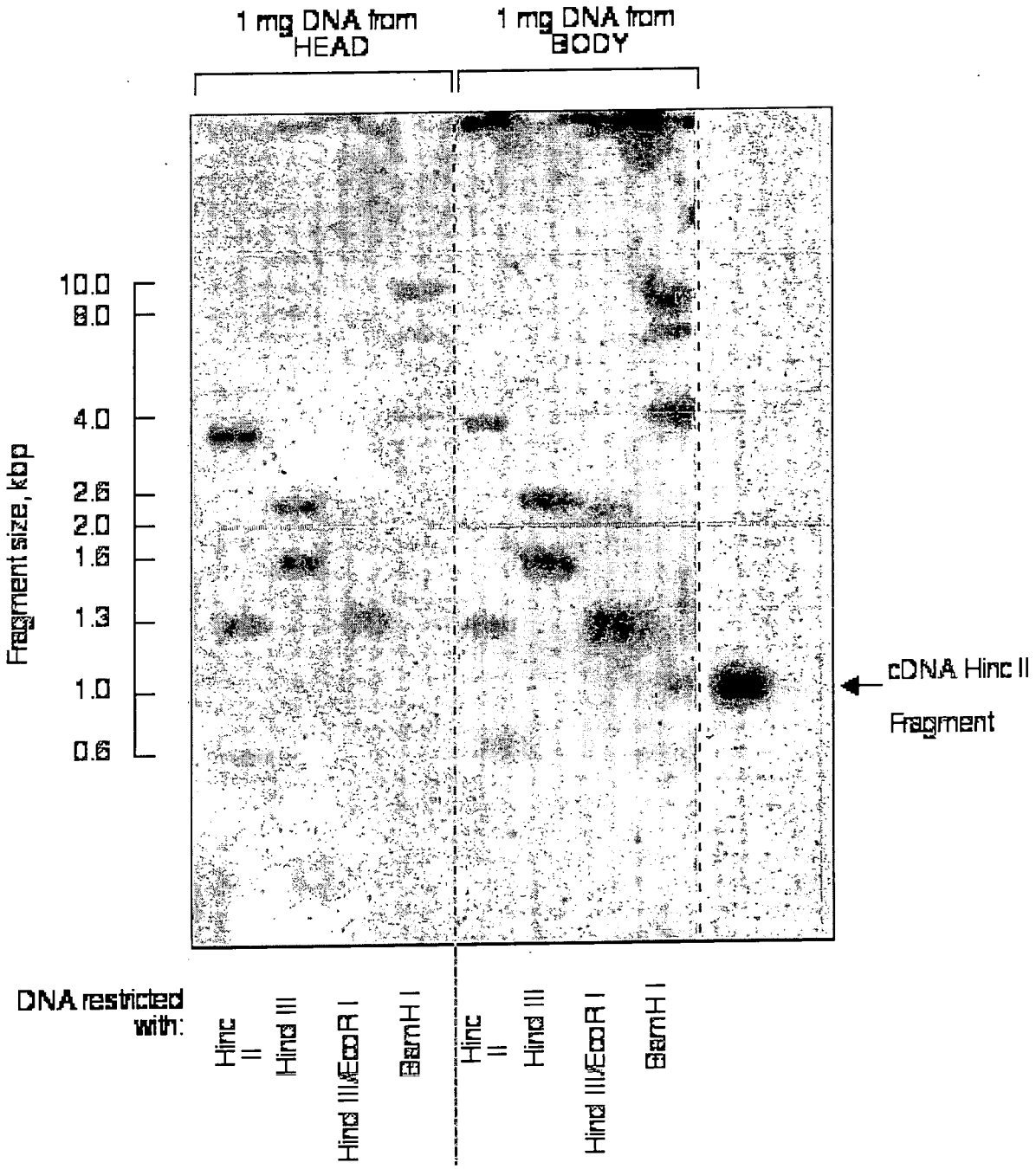


Figure 6-12 Southern Blot of genomic DNA extracted from the heads and bodies of adult vine weevils. The blot was probed with a 1.0Kbp HincII fragment of the VW24 cDNA clone, labelled with 32P-dCTP.

These results clearly show that additional genetic material is located between the restriction sites predicted by analysis of the cDNA.

Enzyme	gDNA from Heads	gDNA from Bodies
Hinc II	4 Kb	4 Kb
	2 Kb	2 Kb
	1.3 Kb	1.3 Kb
	600 bp	600 bp
Hind III	2.6 Kb	2.6 Kb
	1.6 Kb	1.6 Kb
Hind III / EcoRI	2.6 Kb	2.6 Kb
	1.3 Kb	1.3 Kb
BamH I	> 10 Kb	> 10 Kb
	> 8 Kb	> 8 Kb
	4 Kb	4 Kb

Table 6.1 Summary of the restriction fragments identified by the Southern analysis of the genomic DNA isolated from the heads, and the bodies of vine weevil larvae.

6.4.2 Sequencing of Vine Weevil Cellobiohydrolase Introns

To determine the nature of the additional genetic material discovered by the Southern blot a genomic library was constructed from DNA extracted from the heads and bodies, and from the guts of adult vine weevils.

After three sequential rounds of probing the phage library plaque lifts with a probe generated from the same Hinc*II* cDNA fragment as used for the Southern blot a distinct positive plaque was chosen to be sequenced. Restriction digest analysis, using BamH*I*, of the resulting excised plasmid release a 4kbp insert from the multiple cloning site of the cloning vector. Initial sequencing of the insert using vector specific sequencing primers followed by additional sequencing from internal sites gave a contiguous sequence of 3,919 bases.

Alignments between this genomic sequence isolated from the head and bodies of adult vine weevils and the cDNA sequence isolated from the guts of larval weevils shows

that the sequences are homologous. Figure 6-14 shows that these regions of homology are interrupted with segments of DNA only found in the genomic clones; and Figure 6-15 shows a schematic representation of these identified regions. This additional DNA represents the introns found within all eukaryotic genes, and accounts for the band shifts seen in the initial Southern blot.

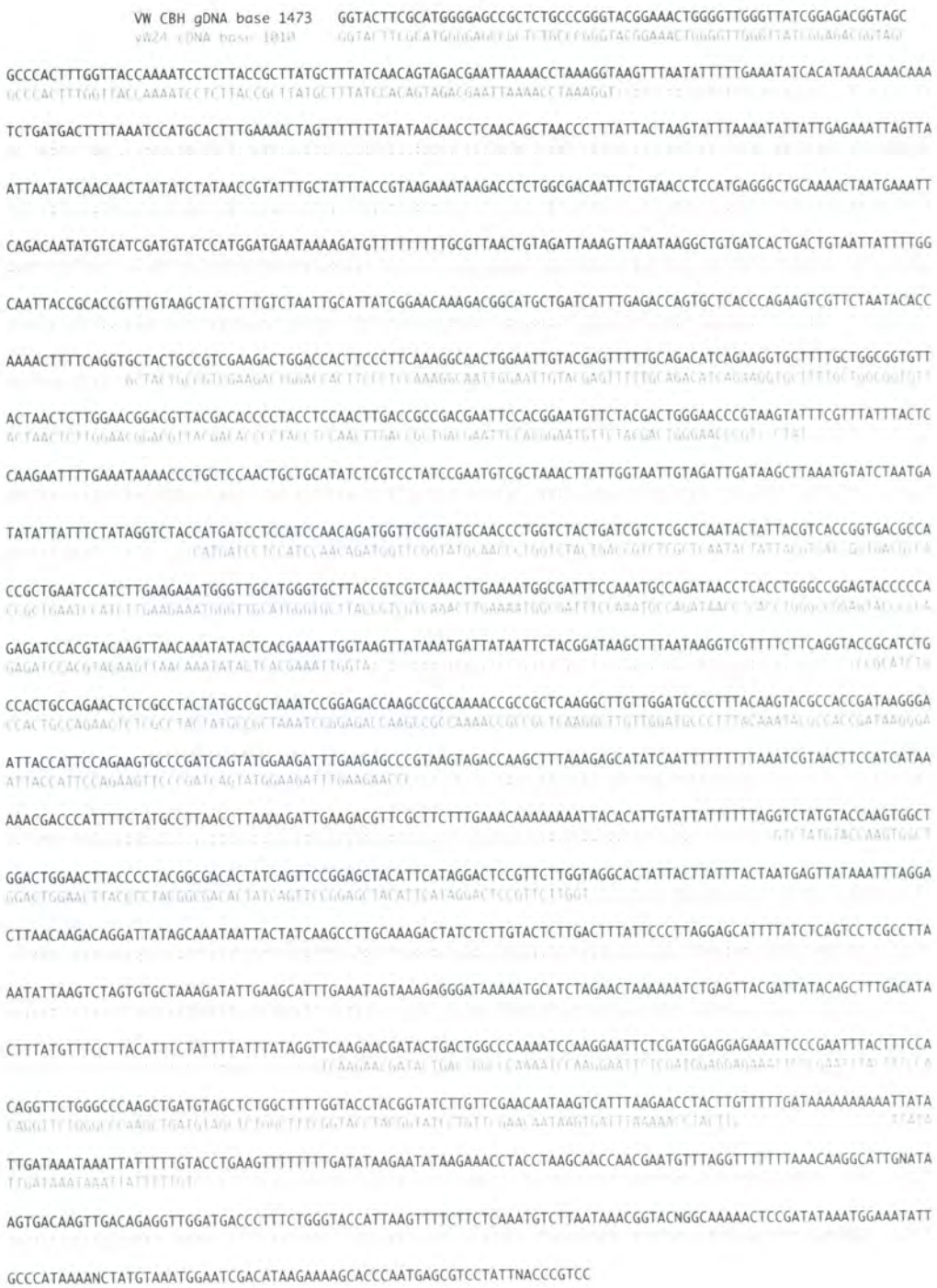


Figure 6-13 Alignment of the partial 3′ VW24 cDNA (blue sequence) isolated from the larval gut cDNA library against the genomic cellobiohydrolase sequence (black sequence) isolated from DNA extracted from the heads and bodies of vine weevil larvae. Spaces in the cDNA sequence indicate the presence of intronic material, many of the intron splice-sites show the characteristic dinucleotide

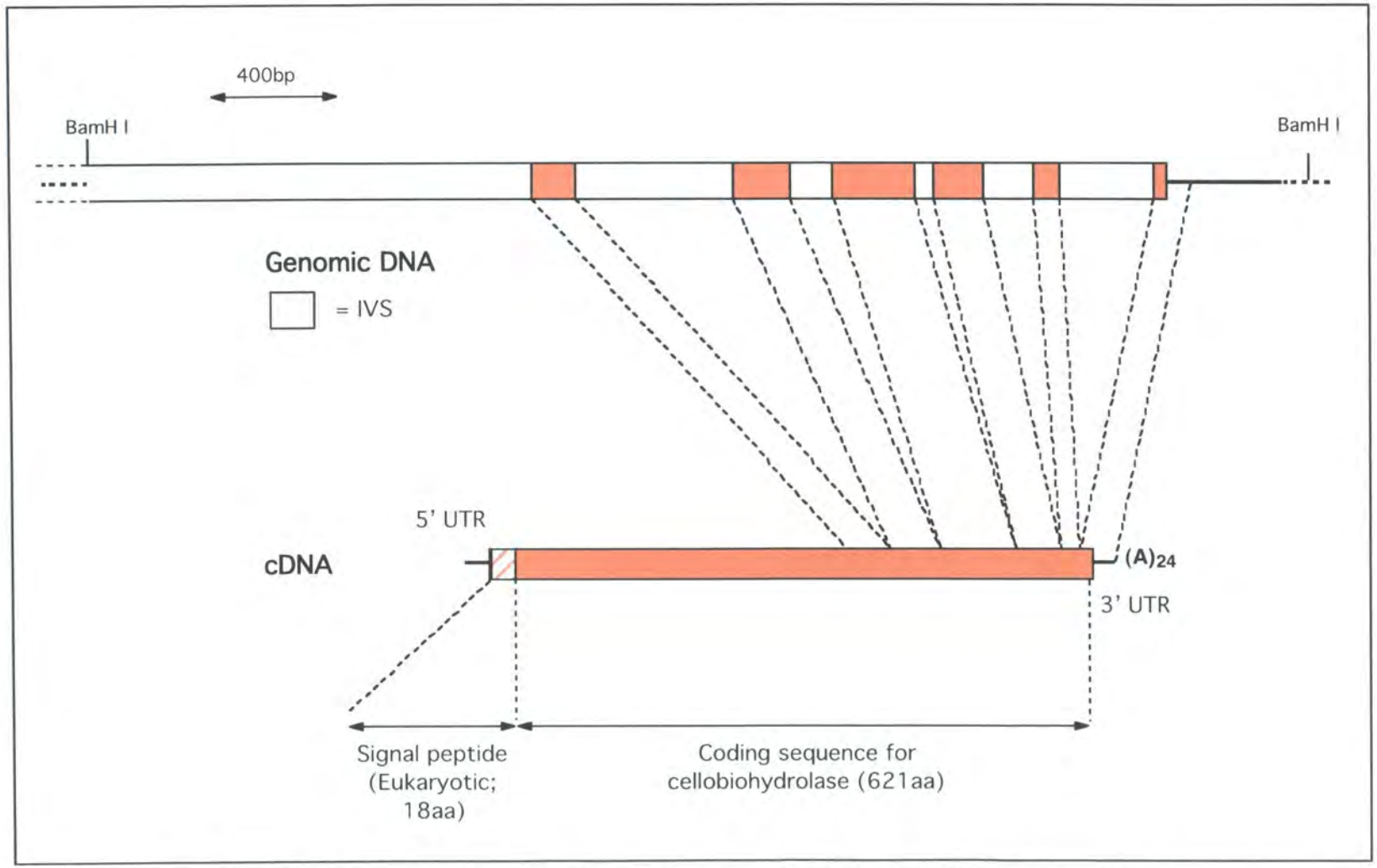


Figure 6-14 cDNA and partial genomic clones encoding vine weevil cellobiohydrolase

6.5 Discussion

Using the technique of 5'RACE it was possible to amplify, clone and sequence the missing information from the 5' end of the original vine weevil cellobiohydrolase cDNA clone, thus completing the ORF, and in addition identifying some 61 bases of the 5' untranslated region. The completed ORF was calculated to contain 1991 base pairs, of which the consensus amino acid sequence shared great levels of homology to known sequences of class 48 glycosyl hydrolases, the cellobiohydrolases. These cellobiohydrolases are secreted exoglucanases typically isolated from bacterial species, as described in Chapter 3 homology searching of the translated protein using BlastP showed greatest similarity to the putative secreted cellulase (family 48) of *Streptomyces coelicolor*. Additional searching with the completed ORF did not change these findings. Figure 6-15 shows a schematic representation of homology alignments between several known cellobiohydrolase enzymes showing the relative positions of the domains present in know CBH-like enzymes. Analysis of known CBH enzymes, and other cellulases, shows a multidomain characteristic with a central catalytic domain flanked by cellulose binding domains at either the N- or C-terminal end. Homology mapping of the vine weevil CBH to those previously identified showed that it did not contain a consensus cellulose binding domain, and was simply a single catalytic domain (Figure 6-15). The clone also contained an endoplasmic reticulum entry signal peptide, which was found to be similar to known eukaryotic sequences.

Biochemical analysis of a crude vine weevil larval gut extract showed activity against a fluorescent cellobiose substrate. When cellobiohydrolase activity was measured over the pH range 3.5 to 10, a peak of activity was observed between pH 4.5 and 6.0. Gut enzyme activity at this pH is concurrent with the cysteine proteolytic activity seen in Chapter 4, indicating actual *in vivo* gut pH , and also with the optimal pH of known cellulases. The evidence presented here strongly supports the hypothesis for the presence of a CBH enzyme within the vine weevil larval gut. Molecular analysis showed that the cDNA clone is curious as it lacks a cellulose binding domain, the supporting biochemical analysis has shown *in vitro* activity of the CBH against a specific synthetic substrate.

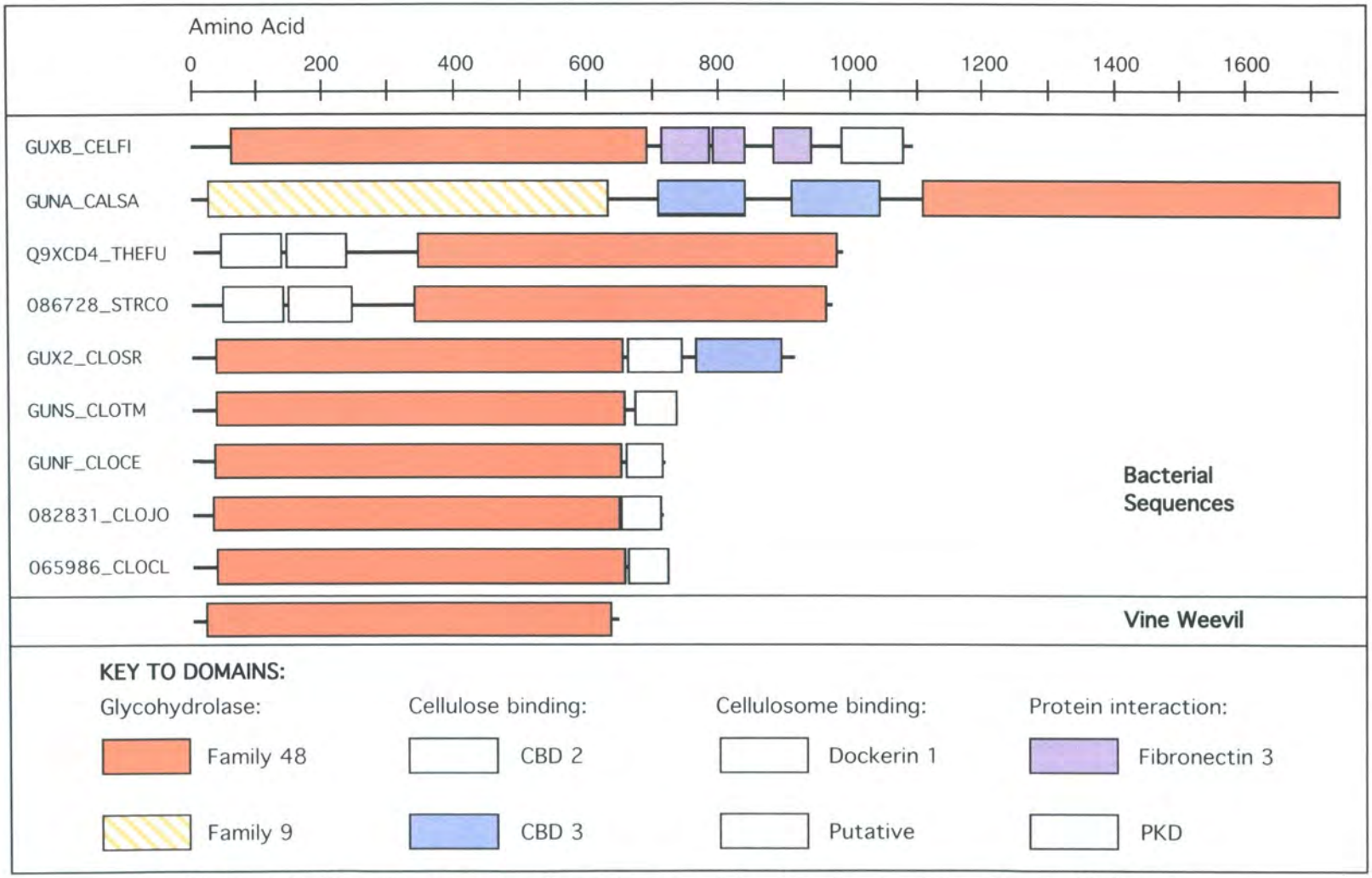


Figure 6-15 Domain structures of proteins containing the glycohydrolase family 48 domain.

6.5.1 Cloning and Expression of Vine Weevil Cellobiohydrolase

The data presented here shows that the clone VW24 isolated from the vine weevil gut cDNA library encodes a functional CBH enzyme as predicted through searching the non-redundant protein sequence database.

6.5.1.1 Expression in a Prokaryotic Host

When the mature coding sequence was introduced into an *E.coli* expression system, full length protein was produced after induction with IPTG. This was shown through comassie stained SDS-PAGE of the proteins eluted from the nickel affinity column. However as Figure 6-8 shows the purification was not efficient enough to give a homogenous sample of the recombinant CBH, with several bands of lower molecular weight appearing on the stained gel. These lower molecular weight contaminants may be from two sources; firstly they may be degradation products of the full-length protein which contain the C-terminal His tag, thus allowing them to be purified from the endogenous *E.coli* proteins, or they may well indeed be endogenous *E.coli* proteins which have been retarded in their migration through the column. I would favour the latter as the purification shown in Figure 6-8 used a low concentration of imidazole (20mM) in the wash buffer, due to it being noted that in earlier experimental purification of the recombinant CBH, the recombinant enzyme was being eluted from the column during the washing process when 60mM imidazole was used (data not shown). This phenomenon may be caused by; masking, or cleavage of the His tag from the recombinant protein, and would account for the co-elution of endogenous *E.coli* proteins. The bacterial expression did however give a protein product of the predicted molecular weight (~66kDa) based on computational analysis of the putative translational product. Although it is clear that recombinant CBH was being expressed in the inclusion bodies of the *E.coli*, no activity was seen against 4-methylumbelliferyl-ß-D-cellobiose after refolding of the denatured protein by dialysis against 0.1M Acetate buffer (pH 5.5). This was due to precipitation of the recombinant protein in an unfolded form from the dialysis solution (seen visually), and/or interactions between the re-folded protein and the cellulose membrane used for the dialysis tubing.

6.5.1.2 Expression in a Eukaryotic Host

A major benefit of expressing recombinant proteins in *Pichia pastoris* is that they can be directed out of the cellular bodies and into the culture medium in a folded active form. Expression of the vine weevil CBH in this was produced active enzyme that could be detected using the 4-methylumbelliferyl-ß-D-cellobiose substrate after precipitation with ammonium sulphate, Figure 6-10. However the recombinant enzyme could not be purified (nickel affinity chromatography) or detected through immuno-blotting (western blotting) via its His tag, leading to the conclusion that it was masked within the folded protein or that it had been removed by proteolytic cleavage. Even though this may be the case, no endogenous CBH activity was seen in assays containing equivalent amounts of *Pichia* culture supernatant from other recombinant lines (*Pichia* expressing a protease inhibitor, pers. comms. D.Bown Fig 5.9), hence all activity seen when using the recombinant CBH culture supernatant can be attributed to the recombinant vine weevil CBH. The recombinant enzyme was finally purified by reversed phase chromatography using phenyl sepharose where the precipitated culture supernatant was loaded in 4M NaCl and eluted in a linear gradient of 4M NaCl to distilled water. Figure 6-9 shows the activities of the load, flow, wash and eluted fractions. In Figure 6-9 it appears as if the eluted fractions is less active than the load fraction, however the reductions in activity is due to an increase in volume of the total eluted fraction over the volume of the load.

Purified enzyme was also shown to be active against total cellulose (CMC) when it was co-polymerised into SDS-PAGE gels. Figure 6-11 shows unstained regions where the cellulose substrate has been removed from the gel by the activity of the recombinant cellobiohydrolase.

6.5.2 Genetic Origin of the Isolated Cellobiohydrolase cDNA

The Southern blot (Figure 6-12) preformed on genomic DNA extracted from the heads and bodies of larval vine weevil clearly shows that when digested with the same restriction enzyme (Hinc*II*) as used to generate the 1kbp probe from the cDNA clone, the resulting bands identified from the genomic DNA are of a different molecular weight to those predicted from the cDNA sequence alone. This additional material must be due to the presence of introns within the vine weevil cellobiohydrolase gene. The blot also shows that when the genomic DNA was digested with a range of

restriction endonucleases the results were different to those expected. These differences are summarised in Table 6.1.

As well as the intronic material increasing the size of the expected fragments, it allows for the additional restriction sites to be included into the sequence and therefore accounts for the presence on the smaller fragments.

By examining the BamH*I* digested gDNA (no BamH*I* site identified in cDNA) the presence of the three bands could suggest that the probe is annealing to three families of the cellobiohydrolase gene. However, the presence of introns means that this conclusion is incorrect.

The additional genetic material was confirmed to originate from the inclusion of introns within the cellobiohydrolase gene by sequencing phagemid clones generated from a BamH*I* digested genomic DNA library. Positive clones were sequenced using both vector specific and sequence specific primers to allow the entire sequence to be confirmed by 'walking' along the clone. Restriction digest analysis using BamH*I* to remove the cloned insert from the vector, revealed the insert to be approximately 4kbp. This size of insert corresponds to the band observed on the Southern blot, and sequence analysis showed the BamH*I* insert to be 3,919 bases.

The second half of the cDNA sequence was found to be wholly contained within the genomic sequence. The two sequences show regions of homology (exons) interrupted with intronic material (Figure 5.13). It is well-established that a majority of intron/exon splice sites conform to consensus sequences. Splicing within these regions exposes dinucleotides at each end of the intron, GT at the 5'end of the intron, and AG at the 3'end. This can be seen with the vine weevil genomic sequence, further indicating that the additional genetic material is intronic in origin.

The evidence of introns within the genomic sequence suggests that the original BCH cDNA sequence was eukaryotic in origin. The eukaryotic origin of the cellobiohydrolase cDNA clone is further highlighted by the unique features that can be identified within it, these features also distinguish it from a sequence of prokaryotic origin.

Domain homology mapping between the vine weevil cellobiohydrolase and exoglucanases from *S. coelicolor*, *C. fimi*, *T. fusca*, and *C. cellulovorans* (Figure 6-15) shows a striking difference in the vine weevil sequence. The map shows that the vine weevil sequence solely consists of a catalytic domain, with no cellulose binding domain (of any origin) being identified. This unique feature of the vine weevil cellobiohydrolase sets it aside from all other known exoglucanases, further enforcing the hypothesis that the cDNA clone is a product of the vine weevil and not from a symbiotic bacteria or fungus. The only other evidence for insect endogenous cellulases is from representatives of the termite families *Termitidae* and *Rhinotermitidae* (Tokuda *et al*, 1999). Tokuda *et al* (1999) used a PCR based method to isolate the genomic cellulase sequence from *N.takasagoensis* which contained 10 exons interrupted by 9 introns, it was further shown that this endoglucanse was specifically expressed in the mature columnar cells of the midgut.

The identification of the partial vine weevil genomic sequence and the published work on termite endogenous cellulases, along with the recent sequencing of an endo-ß-1,4-glucanase from the yellow-spotted longicorn beetle (*P.hilaris*, Sugimura *et al*, 2003) indicates how these insects have evolved to survive on their cellulose rich diets. Although cellulase activity has been observed in several insect orders, in most cases it has not been convincingly established, ie by isolation of genomic sequences and gene expression studies, whether the cellulase activity found in the insect is endogenous or microbial in origin.

The evolutionary origin of these two different classes of cellulase genes in insects can not easily be identified between being relics from earlier metazoan symbionts or as more recent additions to selected groups as a result of a horizontal transfer event. If the latter is true it should open many avenues of thought about the acquisition of novel gene products.

7 General Discussion

The primary goal of this research project was to identify the major classes of enzymes used by the vine weevil during the digestion of ingested food material. Analysis focused upon these enzymes present in in the gut tissue of larval vine weevils whose diet consist mainly of subterranean roots of many plant species. This polyphagous nature of the insect is presumable a result of it's ability to utilise a bank of digestive enzymes to overcome this varied diet. Interestingly the adult vine weevil chooses to eat from aerial regions of the plant, again raising the issue of a further change in the digestive enzyme profile. It was proposed that the information gained would allow for the generation of novel control stratergies towards this pest using GM technology.

The question of identifying these digestive enzymes was answered using two experimental approaches, the first using a molecular biology approach and the second relying upon a upon enzyme characterisation using biochemical analysis. Through the generation of a gut specific cDNA library several clones were identified as mRNA transcripts which encoded for proteins with putative roles in digestion. These clones were identified by randomly screening a small number of clones by way of validating the library and therefore must be abundant within the whole library. The generation of this gut specific cDNA library and the random screening approach has allowed this work to generate a 'snapshot' of the digestive processes occurring within the larval bine weevil. Of those clones sequenced five were shown to have a putative role in digestion, and four more were identified as having cellular 'house-keeping' roles. Additional directed screening of the library also identified two further enzymes from the vine weevil gut. In summary the clones of interest were as follows; two serine protease clones, a cysteine protease, a pectinmethylesterase, a lipase, a chitinase and a cellobiohydrolase.

The protease clones are responsible for the hydrolysis of proteins, the identification of two different classes of proteases indicates that both are utilise by the insect. This was also supported by associated biochemical data that showed the activity of botht eh classes of proteases in their relevant pH conditions. These activities were further characterised through inhibition assays where the activity of each protease was reduced by only it's specific inhibitor. The analysis showed a preference of the lavae to utilise the serine proteases over the cysteine proteases, this is contrary to the observations of Michaud *et al* (1995) who showed that adult VW preferentially use isoforms of cysteine

proteases that are either sensitive or insensitive to the protein protease inhibitor Oc1. This shift in protease usage is presumable a result of a change in the dietary habit of the two life stages of the insect.

The lipase clone isolated from the vine weevil gut may play several roles in the digestive processes of the insect; with similar roles to the mammalian pancreatic lipases in reducing the surface tension of the midgut contents or to generate proline (an essential fuel for activity) from acetyl-CoA in the fat bodies which is derived from the ß-oxidation of fatty acids.

The presence of the pectinmethylesterase clone is an indication of the phytophagous nature of this insect and would be used in the initial process of total degradation of the pectin found in plant cell walls.

The identification of a chitinase cDNA clone from the library gave a basis from which a plant protection scheme could be initiated. The endogenous chitinase of the insect is believed to be responsible for the maintenance of the peritrophic membrane found within the gut lumen. The use of chitinase enzymes as bio-pesticides relies on the assumption that if an insect were to consume a greater amount of active chitinase, the balance of enzyme to peritrophic membrane would be disrupted and thus be deleterious to the insect. The vine weevil chitinase was transformed into, and constiuatively expressed in tobacco plants using the CamV35S viral promoter. Homology comparisons between this chtinase clone and others previously identified from insects shows a novel domain feature of the vine weevil as it contains repeats of the A/SSSSDT motif instead of the more typical PEST region as seen in other chitinases. The PEST region has been implemented in increasing the susceptibility of typical chitinases to proteolysis by calcium-dependent proteases, therefore this work suggests that the chitinase of the vine weevil will have a different susceptibility to this class of protease than those previously described.

The final and most significant of these findings relates to the identification and analysis of a cellulose degrading enzyme. Once the cDNA clone had been identified from the library, activity of the cellobiohydrolase within a extract of the soluble gut proteins was shown against the synthetic fluorescent substrate 4-methylum-belliferylcellobioside. Additional biochemical analysis of the gut extract showed that the vine weevil has the ability to degrade total cellulose. This work also shows that this cellobiohydrolase

enzyme is produced endogenously by the vine weevil and not by any associated gut microflora. The initial Southern analysis used for this identification was later supported by the cloning of a fragment of vine weevil genomic DNA containing regions of the vine weevil cDNA (exons) interspersed with extra genetic material (introns). The evidence of this endogenously encoded cellobiohydrolase gene is the first of it's kind identified in the insect order.

In conclusion the initial objectives of this research project have been largely achieved and it has given an insight into the range of enzymes used by a phytophagous insect in routine digestion. It has also highlighted a change to the current thinking of which organisms are capable of generating cellulose degrading enzymes. This is an area that is ideal for expansion into an examination of these cellulolytic genes in other weevils and also their presence in other phytophagous insects.

8 Annex

8.1 Annex 1: Remaining Sequences Identified Through Random Screening of the Vine Weevil cDNA Library

8.1.1 Clone VW8

Homology searching using the translational product of the predicted ORF found in clone VW8 returned sequences encoding the 40S ribosomal protein S15 as the best match.

```
GTCGTATTCTCTTTACGACAATAAGTAAAAAAATCTTCGTTTTTCGAAGTCATCTGCTGG
V  V  F  S  L  R  Q  .  V  K  K  S  S  F  F  E  V  I  C  W
TAGAATTAATTTAAAATCAAATTTTAATTGCTTAACACCATGGCGGACAAGGGAGAAGAA
.  N  .  F  K  I  K  F  .  L  L  N  T  M  A  D  K  G  E  E
CCGATCAAGAAGAAGAGGACCTTCAAGAAGTTCACTTACAGAGGAGTAGATTTGGACCAA
P  I  K  K  K  R  T  F  K  K  F  T  Y  R  G  V  D  L  D  Q
CTTCTCGATATGCCAAATGAACAACTTATGGAACTAATGCACTGCAGAGCCCGGAGACGT
L  L  D  M  P  N  E  Q  L  M  E  L  M  H  C  R  A  R  R  R
TTCAGTCGCGGTCTAAAACGCAAACCGATGGCCCTCGTAAAGAAGCTCCGCAAAGCCAAA
F  S  R  G  L  K  R  K  P  M  A  L  V  K  K  L  R  K  A  K
AAAGAAGCCCCTCCCCTAGAAAAACCGGAAATCGTCAAGACTCACCTTCGTAACATGATC
K  E  A  P  P  L  E  K  P  E  I  V  K  T  H  L  R  N  M  I
ATCGTTCCCGAAATGGTCGGATCGATCGTAGGAGTCTACAATGGCAAAGCTTTTACCCAA
I  V  P  E  M  V  G  S  I  V  G  V  Y  N  G  K  A  F  T  Q
GTTGAAATCAAACCGGAAATGATCGGCCACTATTTGGGAGAGTTCTCGTTAACATACAAA
V  E  I  K  P  E  M  I  G  H  Y  L  G  E  F  S  L  T  Y  K
CCTGTGAAGCACGGTAGGCCTGGTATCGGTGCCACCCACAGTTCTAGGTTTATTCCTCTT
P  V  K  H  G  R  P  G  I  G  A  T  H  S  S  R  F  I  P  L
AAATAAATGTTTATTTCTCTGCGTTGTATAATTTTAAGGTAAGATGTGATGAAATAAAGG
K  .  M  F  I  S  L  R  C  I  I  L  R  .  D  V  M  K  .  R
TTTTTTTGGAGGAAAA
F  F  W  R  K
```

Figure 8-1 VW8 40S ribosomal protein S15 (23_011299). The underlined region indicates where homology searching identified regions of significant similarity to those sequences of the database.

8.1.2 Clone VW9

Using the standard homology searching method as described earlier the cDNA clone VW9 was identified as encoding the subunit III of cytochrome C oxidase.

```
GCTTTACGAACTTTCCAATACTAATAGGACTAATTAAATGATTTCATTTTCATAACTCAA
F  T  N  F  P  I  L  I  G  L  I  K  .  F  H  F  H  N  S  K
AGTTATTTCTTATTGGTTTATTAATTAATATTTTAATTATATACCAATGATGGCGTGATG
L  F  L  I  G  L  L  I  N  I  L  I  I  Y  Q  .  W  R  D  V
```

```
TCACACGAGAAGGAACCTTTCAAGGACTTCATACTAATAAAGTAGCTCTAGGATTACGAT
T R E G T F Q G L H T N K V A L G L R .
GAGGGATAATCTTATTTATTACATCAGAAATTTTCTTCTTTTTAGCTTTTTTTTGGGGAT
G I I L F I T S E I F F F L A F F W G F
TTCTTCACTCAAGGCTAACCCCTAATATTGAGTTAGGTATTACTTGACCCCCCACAGGA
L H S R L T P N I E L G I T . P P T G I
ATTAAAACATTTAACCCCTTAGAAATTCCCTTATTAAATACCCTTATTCTTTTATCTTCT
K T F N P L E I P L L N T L I L L S S G
GGATTGACTGTTACATGAGCTCATCATAGAATTATAGAAAATGATTATAATCAATCTCT
L T V T . A H H R I I E N D Y N Q S L Q
TCAAGGGTTATCTCTTACTGTCCTTCTAGGATTTTATTTCAGATTATTACAACTTTATGA
G L S L T V L L G F Y F R L L Q L Y E Y
ATATATAGAAGCCCCCTTTACTATCTCAGATAGAGTCTATGGGACCACTTTTTTTATAAC
I E A P F T I S D R V Y G T T F F I T T
AACAGGATTACATGGACTTCATGTGATTATTGGATCTAGATTTCTATTCATTTGCTTATT
G L H G L H V I I G S R F L F I C L L P
ACCGATTATTTTATAACCATCTTTCAAATATTCACCACTTCGGGTTTGAGGCTGCAGCAT
I I L . P S F K Y S P L R V . G C S M I
GATACTGACACTTTGGTGGATGTAGTATGACTATTTTTATACATCCTCATTTATTGATGA
L T L W W M . Y D Y F Y T S S F I D E G
GGGGGGTAATTAAAAAAAAAAAAA
G N . K K K K
```

Figure 8-2 The predicted ORF from clone VW9 (24_011299). The underline regions are those showing significant similarity to cytochrome C oxidase subunit III as identify by searching with Blastx.

8.1.3 Clone VW18

Although the sequencing event of clone VW18 was not very efficient give 'dirty' data with several mis-called or unrecognisable base enough of the consensus sequence was available to allow the cDNA clone to be identified as an acid phosphatase using homology searching to the non-redundant protein database.

```
GTGCATCTCGGCGTTTGGNTGTTNAGGAACAGNTCGTCGNCTACAATNCAGAGATGAAGA
 C I S A F G C ? G T ? R R L Q ? R D E D
TTCATTAGNTTTGGACCATGTGCTTTTTCGTCACGGAAACCGCACCCCTGACAACAACGG
S L ? L D H V L F R H G N R T P D N N G
TTACTCAACCAACCCCATCAACAATGANAGTTTCTATTCGGAGGGCTACTGGCAATTAAC
G Y S T N P I N N ? S F Y S E G Y W Q L
TAATGATGGAAAAAGAACCGAATACAGCATTGGAACAACGCTTCGTNAACGCTACAATCG
T N D G K R T E Y S I G T T L R ? R Y N
CTTCTTAGGNCCGACTTGGAACATCAACTATATCGATGCCAGAACCACCGACACCAACAG
R F L G P T W N I N Y I D A R T T D T N
AACGAAAATGNCTCTTGAACTGATGTTAGCANGACTTTGGCTACCAACGGGCCAACAAAG
R T K M ? L E L M L A ? L W L P T G Q Q
ATGGNTACCTTGGTTGANCTGGNAACCAATACCTTATANCTATTTGGCAAGTGACAAGGA
R W ? P W L ? W ? P I P Y ? Y L A S D K
ACTTCAAAGCACCAGTGTTTGTACAAACTACGATACTGTTGAATGACGAGGTATGAAGAC
E L Q S T S V C T N Y D T V E . R G M K
TCTGACGAAATCCAAGAAGCCTTATCTGNATACAGCGAATATTACGAGTACATGTCTAAT
 T L T K S K K P Y L ? T A N I T S T C L
AAGAGTGGTAAAGATGTCAGCACTCCTTCTAGCGTTTTTGGGCTNTATTTCAGTTTAATT
 I R V V K M S A L L L A F L G ? I S V .
```

```
ACCCAGCTTGAATATGGATATCCACTCGAAGATTGGGTAAAACCAGTATTTCCAGAGGTT
 L  P  S  L  N  M  D  I  H  S  K  I  G  .  N  Q  Y  F  Q  R
ATGGAAAAAATTACC
 L  W  K  K  L
```

Figure 8-3 Clone VW18 (031299_06) identified as an acid phosphatase. The regions of similarity to the know acid phosphatases is shown by the underlined region.

8.1.4 Clone VW14

Blast searching with the predicted ORF from clone VW14 revealed that this clone was homologous to class 2 elongation factors.

```
GTGCATCTCGGCGTTTGGNTGTTNAGGAACAGNTCGTCGNCTACAATNCAGAGATGAAGA
 C  I  S  A  F  G  C  ?  G  T  ?  R  R  L  Q  ?  R  D  E  D
TTCATTAGNTTTGGACCATGTGCTTTTTCGTCACGGAAACCGCACCCCTGACAACAACGG
 S  L  ?  L  D  H  V  L  F  R  H  G  N  R  T  P  D  N  N  G
TTACTCAACCAACCCCATCAACAATGANAGTTTCTATTCGGAGGGCTACTGGCAATTAAC
 G  Y  S  T  N  P  I  N  N  ?  S  F  Y  S  E  G  Y  W  Q  L
TAATGATGGAAAAAGAACCGAATACAGCATTGGAACAACGCTTCGTNAACGCTACAATCG
 T  N  D  G  K  R  T  E  Y  S  I  G  T  T  L  R  ?  R  Y  N
CTTCTTAGGNCCGACTTGGAACATCAACTATATCGATGCCAGAACCACCGACACCAACAG
 R  F  L  G  P  T  W  N  I  N  Y  I  D  A  R  T  T  D  T  N
AACGAAAATGNCTCTTGAACTGATGTTAGCANGACTTTGGCTACCAACGGGCCAACAAAG
 R  T  K  M  ?  L  E  L  M  L  A  ?  L  W  L  P  T  G  Q  Q
ATGGNTACCTTGGTTGANCTGGNAACCAATACCTTATANCTATTTGGCAAGTGACAAGGA
 R  W  ?  P  W  L  ?  W  ?  P  I  P  Y  ?  Y  L  A  S  D  K
ACTTCAAAGCACCAGTGTTTGTACAAACTACGATACTGTTGAATGACGAGGTATGAAGAC
 E  L  Q  S  T  S  V  C  T  N  Y  D  T  V  E  .  R  G  M  K
TCTGACGAAATCCAAGAAGCCTTATCTGNATACAGCGAATATTACGAGTACATGTCTAAT
 T  L  T  K  S  K  K  P  Y  L  ?  T  A  N  I  T  S  T  C  L
AAGAGTGGTAAAGATGTCAGCACTCCTTCTAGCGTTTTTGGGCTNTATTTCAGTTTAATT
 I  R  V  V  K  M  S  A  L  L  L  A  F  L  G  ?  I  S  V  .
ACCCAGCTTGAATATGGATATCCACTCGAAGATTGGGTAAAACCAGTATTTCCAGAGGTT
 L  P  S  L  N  M  D  I  H  S  K  I  G  .  N  Q  Y  F  Q  R
ATGGAAAAAATTACC
 L  W  K  K  L
```

Figure 8-4 Clone VW14 (031299_03) identified as an elongation factor 2 enzyme. The underlined region indicates where homology searching identified regions of significant similarity to those sequences of the database.

8.2 Annex 2: Strategies for Producing Transgenic Plants with Potential resistance to Vine Weevil

8.2.1 Oryzacystatin Expression in Transgenic Tobacco

The generation of plants, which are able to synthesize novel products that can reduce pest infestation and resulting levels of associated damage, has been made available

through developments in plant tissue culture and plant genetic modification. These transgenic plants can be generated by several techniques; here plant transformation using *Agrobacterium* vector system has been used to express the plant derived cysteine protease inhibitor Oryzacystatin *I* (Oc1) in tobacco.

8.2.1.1 Generation of OC1 Plant Expression Vector

The cDNA sequence for the protein protease inhibitor Oc1 had previously been cloned from *Oryza sativa* by Edmonds (1994) and was used as a template for further manipulation. The full length template was amplified by PCR using a pair of primers designed to the terminal 5′ (5′-CCGGATCCATGTCGAGCGACGGAGGGCC-3′), and 3′ (5′-CCGAGCTCTTAGGCATTTGCACTGGCATCGACAGGCTTGAACTCCTGAAGCTC-3′) ends of the sequence. The primers were designed to incorporate unique restriction sites outside of the coding sequence, which would allow directional insertion of the Oc1 ORF as a transcriptional fusion to the promoter of the binary vector (pBI121, ClonTech). Once the fragment had been amplified using Expand polymerase, the gel-purified product was TA cloned into pCR2.1 for sequencing. Sequence analysis showed that there was a substitution event at base 276 where a C had been replaced with a T. This substitution did not however alter the predicted amino acid sequence so the clone was used to generate the expression vector. The coding sequence of Oc1 was digested out of the pCR2.1 vector and ligated into pBI121 before transformation into DH5α to replicate enough recombinant plasmid for transformation into electrocompetent *Agrobacterium tumefaciens* (LBA4404). Putative positive colonies were screened by PCR for the presence of the Oc1 ORF (Figure 8-5), clone LBA4404:OCI.2 was selected for the plant transformation procedure.

8.2.1.2 Tobacco Transformation

Tobacco transformation was performed as described by Gallois and Marinho (1995). This method produced more than thirty primary transformed plantlets, of which twenty were potted-on to be grown to maturity. Once plants were established in the environmentally controlled growth room (16 hour photoperiod at 21°C) leaf samples of approximately 100mg were taken from the forth leaf from the apical bud for analysis. Primary transformed plants (T_0) were covered in breathable 'bread' bags to allow self-

pollination, once dried the second generation (T_1) seeds were collected and stored at 4°C for further usage.

8.2.2 Analysis of T_0 Transformed Tobacco

Two methods were chosen to analyse the expression of the Oc1 ORF in the T_0 transformed plants; the first to look for transcriptional activity, and the second to analyse translation of the protein.

8.2.2.1 RT-PCR of T_0 Transformed Tobacco

Total RNA was extracted from 100mg of leaf tissue (Tri-reagent, Sigma) for lines Oc1-1, Oc1-2 and Oc1-3, of which 1μg was used as a template for RT-PCR analysis. RT-PCR was performed used the same cloning primers used to generate the initial expression construct, Figure 8-6 shows the results of the amplification after electrophoresis in a 2.0% agarose gel. The gel shows amplification of the desired (~320bp) product in all experimental plants (lanes 2,3 and 4) as compared with PCR-based amplification of the positive control pCR2.1:Oc1 (lane 7). No amplification was seen in the two negative controls, lane 5 and 6 (non-transformed tobacco total RNA and no RNA/DNA respectively). The degree of amplification between lanes 2,3 and 4 differs, with plant 2 (lane 3) showing greatest amplification, followed by plant 1 (lane 2) and then plant 3 (lane 4). Due to the fact that each reaction contained 1μg of total RNA this result suggests that plant 2 is transcribing more Oc1 gene product than plant 1 and plant 3. This result confirms that plants 1,2 and 3 had been successfully transformed with the Oc1 ORF and that each had the ability to transcribe the inserted gene to give full length mRNA.

8.2.2.2 Translational Analysis of OC1 T_0 Transformed Tobacco

With the previous result confirming transcription of the inserted Oc1 ORF through molecular based PT-PCR, translation of these messages was analysed by Western Blot used anti-Oc1 antibodies (kind gift of L.Jouanin, INRA).

Total soluble proteins were extracted from 100mg of leaf tissue in leaf extract buffer, and protein content for each sample was estimated by using Bradford reagent, and subsequently 2μg and 4μg of total protein from each plant was separated by SDS-PAGE

using 12.5% polyacrylamide gels. Once electrophoresis was complete the proteins were transferred to nitrocellulose using a semi-dry electroblotter, and plants expressing Oc1 were identified by probing with anti-Oc1 antibodies.

Figure 8-7 shows the result of the Western blot analysis. Lane 1 contains 10ng of purified Oc1 as a positive control and lane 10 contains control non-transformed tobacco, and all other lanes contain experimental plants (plants 1-10 respectively). The Western blot shows that all the experimental plants except plant 8 are expressing the Oc1 gene product. It can also be seen that there are differing levels of expression between each plant line with the highest expression seen in plant 2. This result is consistent with that seen previously with the RT-PCR analysis with both analytical methods showing that between plants 1,2 and 3, plant 2 is producing more Oc1 than plant 1 which is in turn expressing at higher levels than plant 3. The method of Western blotting allows quantification of the amount of Oc1 being expressed by the transformed plants by comparing the intensity of the bands from each expressing line to those of known standard protein concentration. In this case expression levels of between 0.0025% (plant 3) and 0.25% (plant 2) were observed.

8.2.3 Discussion

8.2.3.1 Oryzacystatin Expression in Transgenic Tobacco

The generation of plants engineered to express foreign transgenes has recently become a powerful method of protecting crops from insect pest attack. Using the knowledge gained from Chapter 4 where cysteine protease activity was present in crude extracts of vine weevil gut tissue, and that the activity could be reduced by the addition of specific inhibitors, it was decided to generate several lines of transgenic tobacco expressing the plant derived protein protease inhibitor Oc1.

Once the plants had been transformed with the Oc1 ORF in a CaMV35s/Nos cassette using agrobacteria mediated transformation they were analysed for expression of the transgene. As both Figure 8-6 and Figure 8-7show all but one (90%) of the plants analysed contained the transgene, and were able to express to gene product at levels approaching 0.25% of total soluble leaf protein. The use of two alternate methods of analysis allowed a rapid diagnosis of transcriptional activity for a few experimental lines through RT-PCR (Figure 8-6), followed by translational analysis through Western

blotting of leaf extracts for all 10 lines under investigation (Figure 8-7). Both methods of analysis show a range of expression levels between plant lines. This may be due to several factors such as; the multiple integration of transgene cassettes or the locality of insertion of the cassette into the plant genome. Currently the factors effecting expression levels of transgenes in transgenic plants are difficult to determine, and were beyond the scope of this project.

Although the plants have been shown to express the Oc1 gene product, the effect this would have on vine weevils feeding on the plants could not be tested in this study due to a lack of insect material to perform any meaningful bioassay.

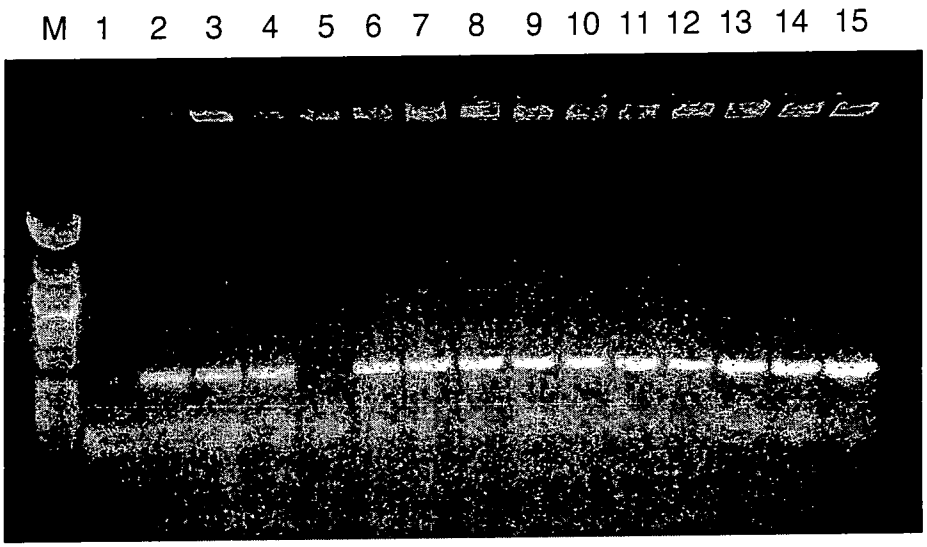


Figure 8-5 Agarose gel (1%) showing products of a colony PCR on putatively transformed *Agrobacterium* LBA4404 to determine insertion of the Oc1 cDNA on the binary vector pBI121 (ClonTech). Lane M; Eco471 molecular weight marker, lane 1; negative control, lanes 2 – 15; putative positive colonies from YEB plates containing 75μg/ml Kanamycin and 100μg/ml rifampicin (lane 2; clone Oc1.1, lane 3; clone Oc1.2 etc.).

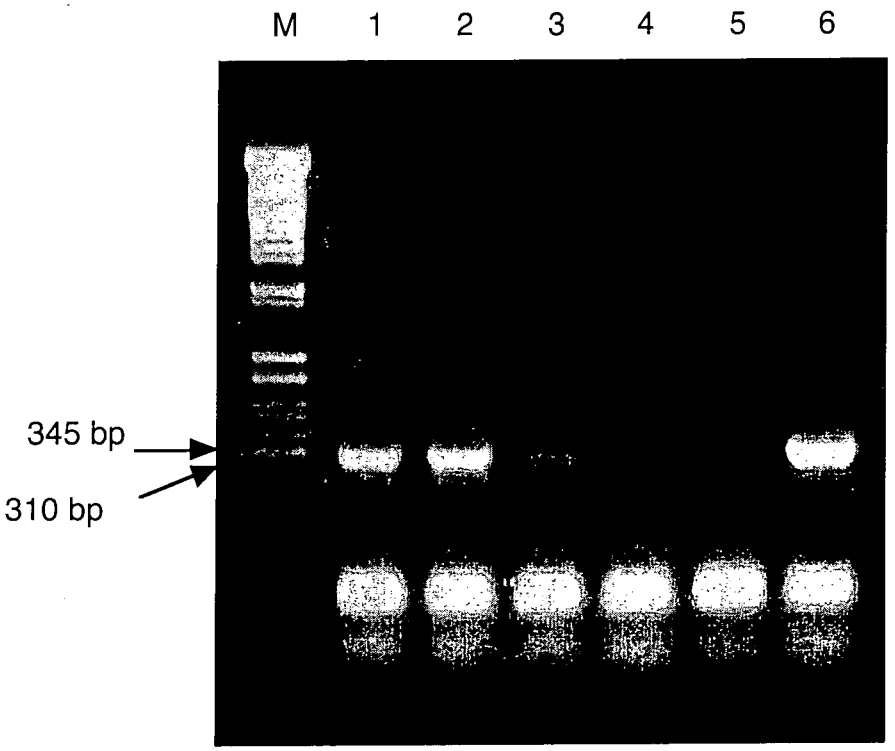


Figure 8-6 Agarose gel (2.0%) showing amplification of a 320 base pair fragment of the Oc1 cDNA, after transformation into tobacco plants. Lane M; Eco471 molecular weight markers, lanes 1, 2 and 3; experimental transgenic tobacco lines expressing Oc1 (1μg total RNA used to initiate reaction), lane 4; 1μg control tobacco RNA, lane 5; no RNA, lane 6; Oc1 cDNA in pCR2.1 (positive control).

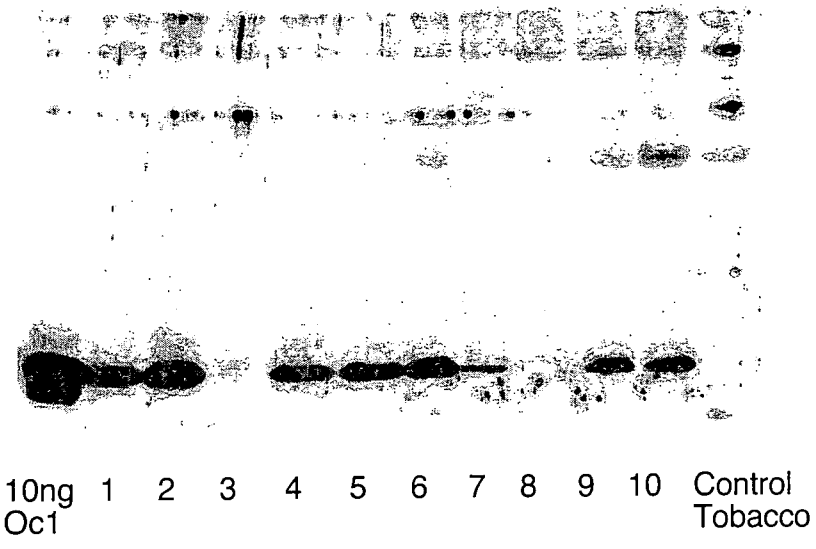


Figure 8-7 Western analysis of primary transformed tobacco containing the Oc1 open reading frame. Lanes 1 through 10 contain 4μg each of total soluble leaf proteins after extraction in 50mM Tris-HCl pH 8.0, 0.1% PMSF. The 15% SDS-PAGE gel was run with 10ng of purified Oc1 (gift from L. Jouanin) and control untransformed tobacco as the appropriate controls (as shown)

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