Development of the Recombinant Aequorin Method and its Evaluation for Calcium Measurement in Filamentous Fungi

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This thesis has been composed by myself. The research of which it is a record was carried out by me. All sources of information have been acknowledged by means of reference.

Glyn Nelson

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Abstract

The aim of this work was to express the Ca^{2+} sensitive photoprotein, apoaequorin, in filamentous fungi to a high level in order to allow routine and simple measurement of changes in cytosolic Ca^{2+} ($[Ca^{2+}]_c$) concentration in living hyphae.

Analysis of the codon bias of the available apoaequorin genes showed a large discrepancy with the codon usage of the target fungi, *Neurospora* crassa, *Aspergillus niger* and *A. awamori*. To overcome this discrepancy, a synthetic apoaequorin gene with optimal codon usage for *Neurospora* and *Aspergillus* was designed and synthesised. An expression vector was generated for the production of apoaequorin from the synthetic gene using a constitutive *Aspergillus* promoter and suitable selectable markers for transformation. An expression vector was also produced for use in *N. crassa* to allow comparison of the aequorin levels obtained between the synthetic gene and the native gene.

Strains of *A. niger* and *A. awamori* were transformed with the *Aspergillus* apoaequorin expression vector, and transformants expressing large amounts of active aequorin were selected and purified. Transformants of *N. crassa* were also produced using the *Neurospora* expression vector, and the highest expression transformants isolated and purified. Comparisons of the aequorin expression levels obtained in these transformants showed a 200-fold higher level of expression in *A. awamori* compared with that obtained in *N. crassa*. Also, the comparison between native and synthetic aequorin production showed a 280-fold higher level in the synthetic aequorin transformant. One high expression transformant from each *Aspergillus* species was chosen for investigation of $[Ca^{2+}]_c$ responses to a variety of external stimuli.

A successful method of growing cultures in small volume, still liquid culture in 96-well microtitre plates was developed which allowed efficient analysis of $[Ca^{2+}]_c$ in

growing cultures. Using such cultures, $[Ca^{2+}]_c$ was monitored during germination, growth and conidiation. A suitable age of culture (18-36 h) was determined for investigation of changes in $[Ca^{2+}]_c$ in response to external stimuli. The first stimulus investigated was the application of a high extracellular concentration (50 mM) of Ca^{2+}, which elicited a large $[Ca^{2+}]_c$ increase. Using suitable Ca^{2+} channel inhibitors, this was shown to be predominantly due to the influx of Ca^{2+} from the media through plasma membrane Ca^{2+} channels.

Investigation of more physiologically significant stimuli was then carried out. The addition of iso-osmotic medium was shown to result in a small, transient rise in $[Ca^{2+}]_c$, which was mainly taken up from extracellular stores. Stimulating cultures with hypo-osmotic medium produced a larger but still transient increase in $[Ca^{2+}]_c$ which also seemed mainly to arise from the influx of Ca^{2+} from an external source. Finally, mycelial $[Ca^{2+}]_c$ increase in response to external pH was shown to be composed of the initial iso-osmotic medium response already characterised, plus a slower secondary response occurring 2 min afterwards. This secondary response increased with greater pH difference, and unlike the medium response was shown to rely primarily upon intracellular Ca^{2+} stores.

Abbreviations

Standard SI units (International System of Units) were used throughout this thesis. Non-SI units and abbreviations are listed below:

ADP	adenosine diphosphate
approx.	approximately
APS	ammonium persulphate
ATP	adenosine triphosphate
BAPTA	1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
$[Ca^{2+}]_c$	cytosolic free calcium concentration
$[Ca^{2+}]_{EXT}$	extracellular free calcium concentration
CaM	calmodulin
CCD	charged coupled device
cDNA	complementary deoxyribonucleic acid
CIAP	calf intestinal alkaline phosphatase
CICR	calcium induced calcium release
CsCl	caesium chloride
dH_2O	distilled water
DEAE	diethylaminoethyl
DAG	diacylglycerol
DMSO	dimethyl sulphoxide
d.NTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis-(ß-aminoethylether)N,N,N',N'-tetraacetic acid

ER	endoplasmic reticulum
EtBr	ethidium bromide
EtOH	ethanol
FDA	fluorescein diacetate
G-protein	guanosine triphosphate binding protein
GDH	glutamate dehydrogenase
GFP	green fluorescent protein
h	hour or hours
HEWL	hen egg white lysozyme
HPLC H_2O	high pressure liquid chromatography purified water
HRP	horse radish peroxidase
IP_3	inositol-1,4,5-trisphosphate
IP_4	inositol-1,3,4,5-tetraphosphate
IPTG	isopropyl ß-D-thiogalactoside
kb	kilobases
kcal	kilo-calorie
kD	kilo-Daltons
LB	luria-bertani
LSD	least significant difference
mcs	multiple cloning site
MeOH	methanol
min	minute or minutes
MOPS	3-[N-morpholino]propanesulfonic acid
mol	mole
M_r	relative molecular weight
mRNA	messenger ribonucleic acid
msec	millisecond or milliseconds
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate reduced
NMR	nuclear magnetic resonance
NP40	octylphenoxy polyethoxy ethanol
nsd	no significant difference

OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI	propidium iodide
PIP2	phosphatidylinositol-4,5-bisphosphate
PK	protein kinase
PLC	phospholipase C
Ponceau S	3-hydroxy-4-[2-sulfo-4(sulfo-phenylazo)phenylazo]-2,7 naphthalenedisulfonic acid
PPT	phosphinothricin
RE	restriction endonuclease
RNA	ribonucleic acid
RNase	ribonuclease
RIP	repeat induced point mutation
rpm	revolution per minute
sec	second or seconds
SD	standard deviation (n-1)
sd	significant difference
sd H_2O	sterile distilled water
SDS-PAGE	sodium dodecyl sulphate - polyacylamide gel electrophoresis
TEMED	N,N,N',N-tetramethylethylenediamine
tRNA	transfer ribonucleic acid
TFP	trifluoperazine
Tween 20	polyoxyethylene sorbitan monolaurate
UV	ultra-violet
VS	1 x Vogels medium N + 1% sucrose
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

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1. INTRODUCTION

1.1 Eukaryotic Signal Transduction

Recognising external signals and reacting correctly to them is an important function for all cells. Eukaryotes have developed complex signalling systems to relay and interpret external physiochemical and biochemical stimuli. These systems utilise specific receptors on the cell surface, which interact with different intracellular signalling pathways to produce a range of specific responses. Intracellular signalling pathways include kinase cascades, and pathways mediated by the second messengers cyclic-adenosine monophosphate (cAMP), cyclic-guanosine monophosphate (cGMP), and Ca^{2+}. These intracellular pathways all act to amplify and diversify the stimulus and produce a specific cellular responses to a specific stimulus. There is evidence for all of these intracellular signalling pathways operating in filamentous fungi (Gadd, 1994).

1.1.1 Evidence for Ca^{2+} as a second messenger

The role of calcium as a second messenger has been investigated in substantial detail in eukaryotes, especially in mammalian cells (for reviews see Berridge and Dupont, 1994; Berridge, 1995a). Most of the components of the signal transduction pathways appear to be very similar in all eukaryotic systems, and thus much of what has been discovered in animal and plant cells, and in the budding yeast *Saccharomyces cerevisiae*, probably also applies to filamentous fungi (Gadd, 1994).

Table 1.1: Examples of evidence for the involvement of Ca^{2+} signalling in various fungi

Species	Evidence for Ca^{2+} use	Reference
Neurospora crassa	decrease in $[Ca^{2+}]_c$ gives loss of apical dominance	(Schmid and Harold, 1988; Dicker and Turian, 1990)
	inhibition of circadian conidiation rhythm by Ca^{2+} ionophore and CaM inhibitors	(Nakashima, 1984; Nakashima, 1986; Sadakane and Nakashima, 1996)
	CaM-mediated phosphorylation required for conidial germination	(Rao *et al.*, 1997)
Aspergillus nidulans	Ca^{2+}-CaM required for cell cycle	(Lu *et al.*, 1992; Lu *et al.*, 1993)
Aspergillus fumigatus	low external $[Ca^{2+}]$ causes hyperbranching	(Gow *et al.*, 1992)
Ophiostoma ulmi	role of Ca^{2+} in fungal dimorphism	(Muthukumar and Nickerson, 1984)
	role of Ca^{2+} and CaM in fungal dimorphism	(Gadd and Brunton, 1992)
Trichoderma viride	$[Ca^{2+}]_{EXT}$ required for conidiation	(Krystofova *et al.*, 1996)
Saccharomyces cerevisiae	role of Ca^{2+} in cell cycle control	(Anraku *et al.*, 1991; Eilam and Chernichovsky, 1987)
	response to hypotonic shock requires $Ca^{2+}{}_{EXT}$	(Batiza *et al.*, 1996)
	Ca^{2+} channels involved in mating	(Fischer *et al.*, 1997)
Fusarium graminearum	$[Ca^{2+}]_{EXT}$ and Ca^{2+} inhibitors affect hyphal extension and branching	(Robson *et al.*, 1991a; Robson *et al.*, 1991b)
Candida albicans	requirement for Ca^{2+}-CaM in dimorphism	(Roy and Datta, 1987; Sabie and Gadd, 1989)
Pythium spp.	effects of Ca^{2+}, Ca^{2+}-modulators and CaM antagonists upon zoospore motility	(Deacon and Donaldson, 1993; Donaldson and Deacon, 1993)
Phytophthora infestans	increased CaM levels upon infection of host	(Pieterse *et al.*, 1993)
	$[Ca^{2+}]_{EXT}$ required for germination	(Hill *et al.*, 1998)
Phytophthora parasitica	$[Ca^{2+}]_{EXT}$ requirement for encystment of zoospores and internal Ca^{2+} store requirement for cyst germination	(Warburton and Deacon, 1998)

Key: CaM, calmodulin (a ubiquitous Ca^{2+} binding protein). $[Ca^{2+}]_{EXT}$, external Ca^{2+}

Ca^{2+} has been shown to be involved in intracellular signal transduction arising from various stimuli in filamentous fungi and shown to be important in regulating essential activities such as hyphal tip growth (Jackson and Heath, 1993), regulation of branching, zoospore motility and circadian rhythm. Examples of involvement of Ca^{2+} in these processes is given in Table 1.1. Growing evidence suggests that a tip

high cytosolic free calcium gradient in hyphae is required for polarized tip growth (Table 1.1, and reviewed in Gow, 1994).

1.1.2 Overview of Ca^{2+} signal transduction pathway

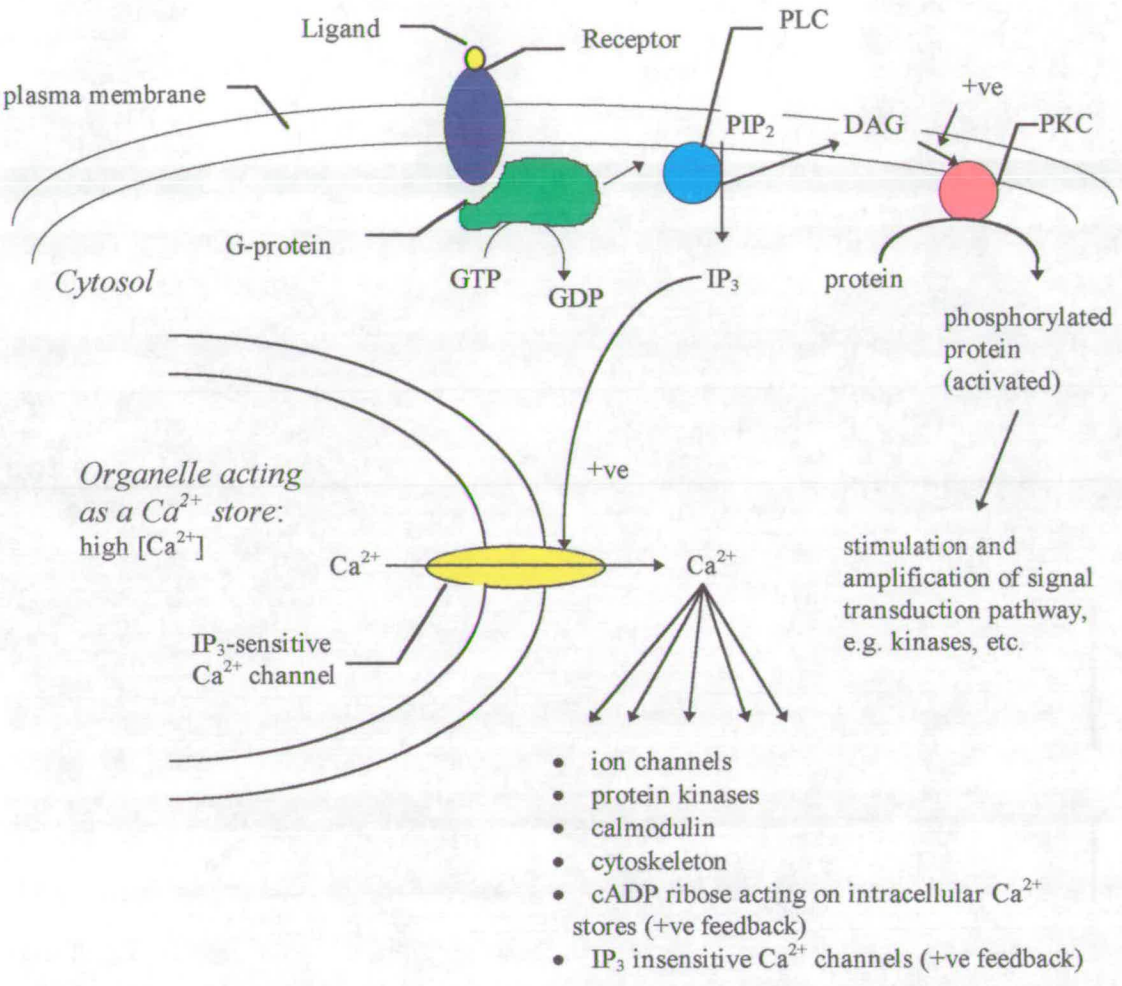
1.1.2.1 Calcium as a second messenger in fungi

The normal 'resting' level of cytosolic free calcium ($[Ca^{2+}]_c$) in filamentous fungi is approximately 100-300 nM (Miller *et al.*, 1990). The cell receives stimulation through activation of receptor molecules which may be specific to the stimulus. Upon excitation of the cell by a suitable stimulus there is an increase in $[Ca^{2+}]_c$ which may be localised (as a 'spike' or 'spark') at the point of excitation. This Ca^{2+} spike can then spread across the cell as a wave, eliciting a response, which will ultimately modulate translation or transcription of specific genes to allow a specific response to the stimulus (Berridge, 1993b; Berridge and Dupont, 1994). The level of $[Ca^{2+}]_c$ increase depends upon the strength of the stimulus (Bootman *et al.*, 1994). Most stimuli are extracellular and are mediated via a plasma membrane bound receptor (e.g. response to hormone, Bootman *et al.*, 1996). Membrane bound receptors are predominantly G-protein linked. In response to stimulation, $[Ca^{2+}]_c$ is increased either from cell entry of Ca^{2+} through plasma membrane Ca^{2+}-channels, or through Ca^{2+} release from internal cell compartments acting as Ca^{2+} stores (Berridge, 1997). High levels of $[Ca^{2+}]_c$ are toxic to the cell due to its reaction with phosphate-containing compounds (Campbell, 1983), and therefore free Ca^{2+} is quickly removed either from the cell or into Ca^{2+}-containing organelles (*e.g.* vacuole) by active transport. In *Neurospora crassa*, the vacuole has been shown to play an important role for storage of Ca^{2+} (Cornelius and Nakashima, 1987). Release of Ca^{2+} from internal stores can occur through channels which are opened by inositol-1,4,5-triphosphate (IP_3), (Cornelius *et al.*, 1989). IP_3 has also been shown to be involved in uptake of extracellular Ca^{2+}, thought to be required for refilling intracellular stores (Bennett *et al.*, 1998). IP_3 is the water-soluble breakdown product of phosphatidyl-inositol 4,5-bisphosphate (PIP_2), which is cleaved by phospholipase C in the plasma

membrane. Diacylglycerol (DAG), the hydrophobic breakdown product of PIP_2, activates membrane-bound protein kinase C through the plasma membrane. PIP_2 cleavage occurs in response to an activated receptor in the plasma membrane stimulating a membrane bound G-protein which dissociates into the subunits G_α and $G_{\beta\gamma}$, both of which activate isozymes of phospholipase C (Figure 1.1).

Cyclic adenosine 5'-diphosphoribose (cADP ribose) is a metabolite of NAD^+ and has recently been shown to act as an agonist for Ca^{2+} induced Ca^{2+} release in a similar manner as IP_3 (Galione, 1993; Lee, 1994). It has been shown to release Ca^{2+} from ryanodine receptors through calmodulin in sea urchin egg microsomes (Lee *et al.*, 1994), and has been shown to operate as a second messenger in red beet microsomes (Muir and Sanders, 1996) and is involved in stomatal closure (Leckie *et al.*, 1997).

Figure 1.1: Outline model of Ca^{2+}-IP_3 signalling



Key: PLC- phospholipase C, PIP_2- phosphotidylinositol 4,5-bisphosphate, DAG- diacylglycerol, IP_3- inositol 1,4,5- triphosphate, PKC- protein kinase C. Binding of ligand to receptor changes the conformation of the receptor, allowing binding of G-protein. This stimulates the displacement of GDP by GTP on the α- subunit of the G-protein. This activates the α- subunit, which in turn activates PLC, to cleave PIP_2 into DAG and IP_3. DAG stimulates PKC to phosphorylate various regulatory proteins, and IP_3 moves into the cytosol, where it can interact with free Ca^{2+} and open IP_3- sensitive Ca^{2+} channels on internal Ca^{2+} storing organelles, thus mobilising intracellular Ca^{2+}. (Adapted from Gadd, 1994)

1.1.3 Ca^{2+} channels

Rapid flow of Ca^{2+} from extracellular and intracellular sources occurs through Ca^{2+} channels because the ions are moving down the transmembrane electrochemical gradient. $[Ca^{2+}]_c$ is normally kept at a low resting level by means of Ca^{2+} pumps which transport Ca^{2+} up a concentration gradient either across the plasma membrane or across membranes of organelles which store Ca^{2+} (Calvert and Sanders, 1995).

1.1.3.1 Maintenance of $[Ca^{2+}]_c$

Ca^{2+}/H^+ antiport transporters in the plasma membrane pump Ca^{2+} out of the cytosol in exchange for H^+ (Stroobant and Scarborough, 1979). It is thought that these transporters are ATP-dependent pumps with a stoichiometric ratio of 2 H^+ per Ca^{2+} (Miller *et al.*, 1990).

In the vacuole, Ca^{2+}/H^+ antiport systems transport Ca^{2+} across the membrane, and the proton gradient across the vacuole is maintained by H^+-ATPase pumps (Klionsky *et al.*, 1990; Anraku *et al.*, 1991), which also help to control cytosolic pH. Several stoichiometries have been suggested for plant vacuolar Ca^{2+}/H^+ antiports, with $3H^+$ per Ca^{2+} being the most likely (Blumwald and Gelli, 1997). However, this cannot be strongly relied upon as the presence of tonoplast Ca^{2+}-ATPases could affect the calculation (Blumwald and Gelli, 1997). Such transporters have been recorded in plant tonoplast (Chanson, 1993), so interference from these is a possibility. The isolation of two genes from *Arabidopsis thaliana* thought to encode vacuolar H^+/Ca^{2+} antiports (Hirschi *et al.*, 1996) will allow a more thorough investigation of the structure/function characteristics of these carriers. Mutants of *S. cerevisiae* lacking a functional gene for a vacuolar H^+-ATPase are not able to control $[Ca^{2+}]_c$ (Ohya *et al.*, 1991), showing the importance of maintaining the proton gradient and the importance of the vacuole in Ca^{2+} storage. There is also evidence for a Ca^{2+}-ATPase in the vacuolar membrane of *S. cerevisiae* (Cunningham and Fink, 1994) and a H^+/Ca^{2+} antiport which is inactivated by calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase (Cunningham and Fink, 1996). Conservation of

vacuolar Ca^{2+} transporters between species appears to be very high, as H^+/Ca^{2+} antiports from *A. thaliana* are operable in *S. cerevisiae* (Hirschi *et al.*, 1996), as are putative endoplasmic reticulum (ER) Ca^{2+}-ATPase pumps of *A. thaliana* (Liang *et al.*, 1997). The importance of the vacuole for Ca^{2+} homeostasis is highlighted, as it allows fungal cells to grow in media containing a $[Ca^{2+}]$ as high as 100 mM $CaCl_2$ (Ohya *et al.*, 1986).

A possible ER Ca^{2+}-ATPase has been found in *Schizosaccharomyces pombe* (Ghislain *et al.*, 1990). This is encoded by the *cta3* gene, which most closely resembles mammalian ER Ca^{2+}-ATPase genes. By inhibiting vacuolar Ca^{2+} uptake (using a protonophore to uncouple the proton gradient) and permeabilising the plasma membrane (using nystatin), the *cta3* gene product was shown to be a non-vacuolar, internal ATP-dependent Ca^{2+} channel (Halachmi *et al.*, 1992). Residual ATP-dependent Ca^{2+} uptake was observed in the *cta3* null mutant, suggesting that another non-vacuolar Ca^{2+}-ATPase exists (Halachmi *et al.*, 1992).

There is very little evidence to suggest that mitochondria are important organelles in regulation of $[Ca^{2+}]_c$ (Pietrobon *et al.*, 1990). Mitochondrial Ca^{2+} transport is thought to be important for some intra-mitochondrial reactions, but *S. cerevisiae* Ca^{2+}-sensitive mutants do not show mitochondrial defects (Ohya *et al.*, 1991).

All of the channels and pumps mentioned above act to maintain the resting level of $[Ca^{2+}]_c$ and to regulate cytosolic pH, whilst being able to accumulate Ca^{2+} in organelles which can be released quickly upon stimulation.

1.1.3.2 Extracellular Ca^{2+} uptake and intracellular Ca^{2+} release

Ca^{2+} influx from the medium is via ligand- or voltage-gated channels, and occurs by passive movement of Ca^{2+} down the electrochemical gradient (Pietrobon *et al.*, 1990). Also, a plasma membrane mechanosensitive (MS) channel which allows several cations (including Ca^{2+}) into the cell has been shown to exist in *Uromyces*

appendiculatus, which is thought to convert membrane stress into an influx of ions as a signal initiation for the stress response (Zhou *et al.*, 1991). MS Ca^{2+} channels have been recorded using patch clamping in *N. crassa* (Levina *et al.*, 1995) and the oomycete *Saprolegnia ferax* (Garrill *et al.*, 1992). Differences in channel position and function have been observed between the two species. *S. ferax* MS Ca^{2+} channels are located with a tip high gradient which is apparently maintained by interaction with F-actin in the cytoskeleton (Levina *et al.*, 1994). *N. crassa* hyphae do not seem to exhibit a gradient in channel placement, although the channels are seen to cluster together (Lew, 1998). Both species have a tip high $[Ca^{2+}]_c$ gradient which is essential for growth (Garrill *et al.*, 1993; Levina *et al.*, 1995). In *S. ferax* the MS Ca^{2+} channels are required to maintain this gradient (Garrill *et al.*, 1993), but they are not involved in maintaining the gradient in *N. crassa*. Intracellular stores in *N. crassa* may play a role in producing the tip high $[Ca^{2+}]_c$ gradient (Levina *et al.*, 1995).

Release from intracellular stores is also passive down a concentration gradient of Ca^{2+}. Gated channels are predicted to exist which allow release of calcium in response to specific stimuli. Plant vacuoles are known to have voltage- and IP_3-gated Ca^{2+} channels (Johannes *et al.*, 1991; Johannes *et al.*, 1992a; Alexandre and Lassalles, 1992), and IP_3-gated Ca^{2+} channels have been shown in *N. crassa* (Cornelius and Nakashima, 1987; Cornelius *et al.*, 1989).

1.1.3.3 Ca^{2+}-binding proteins

As shown in Figure 1.1, increased $[Ca^{2+}]_c$ may bind to and directly regulate the activity of many proteins, such as ion channels and regulatory proteins (e.g. kinases, phosphatases, adenylate cyclases, etc.). This interaction may be by direct Ca^{2+} binding to the regulatory protein or via the primary intracellular Ca^{2+}-binding protein, calmodulin (CaM). CaM appears to be ubiquitous throughout eukaryotes. CaM genes have been found in various fungi, including *Candida albicans* (Saporito and Sypherd, 1991), *S. cerevisiae* (Davis *et al.*, 1986), *N. crassa*, (Cox *et al.*, 1982),

and *S. pombe*, (Takeda and Yamamoto, 1987). The amino acid sequence of CaM is highly conserved among eukaryotes, with plant CaM sequences generally showing >90% homology with bovine brain CaM, and fungal CaM sequences showing 60-80% homology with bovine brain CaM. Such close similarities between diverse eukaryotes reflect the essential role of CaM. CaM is required for cellular functions such as nuclear division, cell cycle control, and cell proliferation (Anraku *et al.*, 1991). Elevated $[Ca^{2+}]_c$ increases the binding of Ca^{2+} to CaM, causing conformational change in the protein, which increases its affinity for binding to various CaM target proteins, and in so doing modifying the activity of these proteins. There is evidence however, through site-directed mutagenesis of the yeast CaM gene, that CaM can successfully carry out some of its roles without binding Ca^{2+} (Geiser *et al.*, 1991).

The interaction of CaM with adenylate cyclase is a strong link between the Ca^{2+} and cAMP signalling pathways (Reig *et al.*, 1984; Panchenko and Tkachuk, 1984). Also, CaM is known to be involved in regulating cyclic-adenosine diphosphate ribose (cADP-ribose) interaction with ryanodine receptors (Lee *et al.*, 1994). Such interactions reinforce the view that the signalling transduction mechanisms within a cell are closely linked and form an interconnecting network of signalling pathways.

1.1.4 Formation of the Ca^{2+} response signal

1.1.4.1 Role of intracellular Ca^{2+} stores

The initial local increase in $[Ca^{2+}]_c$ after stimulation does not spread quickly because Ca^{2+} diffusion in the cytosol is very slow. Increased $[Ca^{2+}]_c$ acts with IP_3 as an agonist upon the IP_3-sensitive Ca^{2+} channels, further increasing $[Ca^{2+}]_c$ (Berridge and Dupont, 1994). IP_3-sensitive Ca^{2+} channels are located in the ER membrane in animal cells and in the tonoplast of plants and fungi (Cornelius *et al.*, 1989; Johannes *et al.*, 1991; Alexandre and Lassalles, 1992). The IP_3 receptor becomes more

sensitive to cytosolic Ca^{2+} when IP_3 concentration increases, switching to a low affinity state above 300 nM IP_3 (Berridge, 1993b).

Released Ca^{2+} also interacts with IP_3-insensitive Ca^{2+} channels to release Ca^{2+} from IP_3-insensitive stores (Berridge and Irvine, 1989; Nakajima-Shimada *et al.*, 1991). Ryanodine receptors are internal IP_3-insensitive Ca^{2+} channels, and have been shown to exist in several mammalian cell types, such as muscle cells and neurons (McPherson and Campbell, 1993a; McPherson and Campbell, 1993b). Ryanodine receptors have been shown to release Ca^{2+} upon stimulation with the putative second messenger, cyclic ADP-ribose (Berridge, 1993a), and Lee *et al.* (1994) suggested a role for CaM in modulating ryanodine receptor sensitivity to cyclic ADP-ribose. Release of intracellular Ca^{2+} which is controlled by the local $[Ca^{2+}]_c$ levels is known as 'calcium-induced calcium release' (CICR, Berridge, 1993a). Also, there is evidence that IP_3 allows Ca^{2+} influx from outside the cell (Berridge, 1993a). This may be carried out after conversion of IP_3 to inositol 1,3,4,5-tetraphosphate, (IP_4) by 3-kinase, and may also regulate the refilling of intracellular Ca^{2+} pools (Berridge and Irvine, 1989). Both these pathways change the initial localised spike of Ca^{2+} release into a diffusing wave of Ca^{2+}, which spreads throughout the cell. This is not necessarily smooth, and may be seen as oscillations of Ca^{2+} across the cell (Berridge and Irvine, 1989). The peaks of $[Ca^{2+}]_c$ in the oscillations occur due to the wave of Ca^{2+} reaching another Ca^{2+}-storage organelle, causing a further spike of release from the store in that area of the cell (Berridge and Dupont, 1994). Ca^{2+} waves are not restricted to individual cells, but have been shown to be transmitted across tissue and organs in several organisms (Cubitt *et al.*, 1995; Knight *et al.*, 1993a; Osipchuk and Cahalan, 1992), with secreted ATP or cAMP as the suggested transmission signals. Cells which are cytoplasmically connected will be able to transmit the wave with $[Ca^{2+}]_c$, or via other second messengers such as IP_3 or cADP-ribose.

The IP_3-sensitive Ca^{2+} pool is thought to be refilled by capacitative Ca^{2+} entry (CCE), which is driven by the Ca^{2+} concentration within the ER causing influx of

extracellular Ca^{2+} (Berridge, 1996). The hypothesis for CCE requires transmission of information from the ER to the plasma membrane. Several methods have been proposed for passage of this signal, including use of G-proteins, conformational coupling and protein phosphorylation (Berridge, 1995b). Conformational coupling is thought to be the central method for signal transmission, via the N-terminal domain of the ER IP_3 receptor to the plasma membrane Ca^{2+} channels, either directly or through the cytoskeleton (Berridge, 1995b). For this model to be correct, the ER must be closely associated with the plasma membrane. Use of cell fractionation and electron microscopy have revealed this to be the case (Rossier *et al.*, 1991; Chadwick *et al.*, 1992). Studies using mammalian PC12 cells have suggested that the IP_3-sensitive stores may control the initial uptake of extracellular Ca^{2+} (Bennett *et al.*, 1998). Bennett *et al.* also showed that IP_3-sensitive stores, ryanodine receptor stores and thapsigargin-sensitive stores all required a common component of the CCE pathway as stimulation of one store subsequently diminished the affect of stimulating another store. However, the different stores did produce an additive CCE response when emptied, suggesting that they are independent. Recent studies have shown the presence of a diffusible 'calcium influx factor' in the cytosol of cells whose intracellular Ca^{2+} stores have been depleted (Csutora *et al.*, 1999). Partially purified extracts from such cells elicited an increase in $[Ca^{2+}]_c$ which was dependent on extracellular Ca^{2+} and independent of IP_3. This factor was also shown to be functionally conserved between human T lymphocytes and yeast, as extracts from both elicited a response in *Xenopus laevis* oocytes (Csutora *et al.*, 1999).

In summary, the Ca^{2+} signalling pathway appears to be conserved throughout eukaryotes, with the largest difference appearing to be use of the vacuole in plants and possibly fungi as the major intracellular Ca^{2+} store, compared to the use of the ER as the primary Ca^{2+} store in mammalian cells. It is also clear in animal cells that other organelles can act as Ca^{2+} stores (Rizzuto *et al.*, 1995). Studies so far suggest that the mammalian ER and plant vacoule both contain similar Ca^{2+} channels and that they appear to operate in a very similar way. Recent research underlines the

complicated integration of different signalling pathways within cells, with other second messengers such as IP_3, cAMP and cADP-ribose affecting Ca^{2+} signalling. Overall, the second messengers appear to produce an integrated signalling network across the cytosol of cells. This increases the difficulty of analysing and defining the role of one second messenger alone.

1.2 *In vivo* measurement of $[Ca^{2+}]_c$

To successfully investigate the role of Ca^{2+} within fungal cells, *in vivo* measurement is required (Read *et al.*, 1992). Ways of measuring $[Ca^{2+}]_c$ include using Ca^{2+}-sensitive fluorescent dyes, and also Ca^{2+}-sensitive bioluminescent proteins, such as aequorin.

1.2.1 Ca^{2+}-sensitive fluorescent dyes

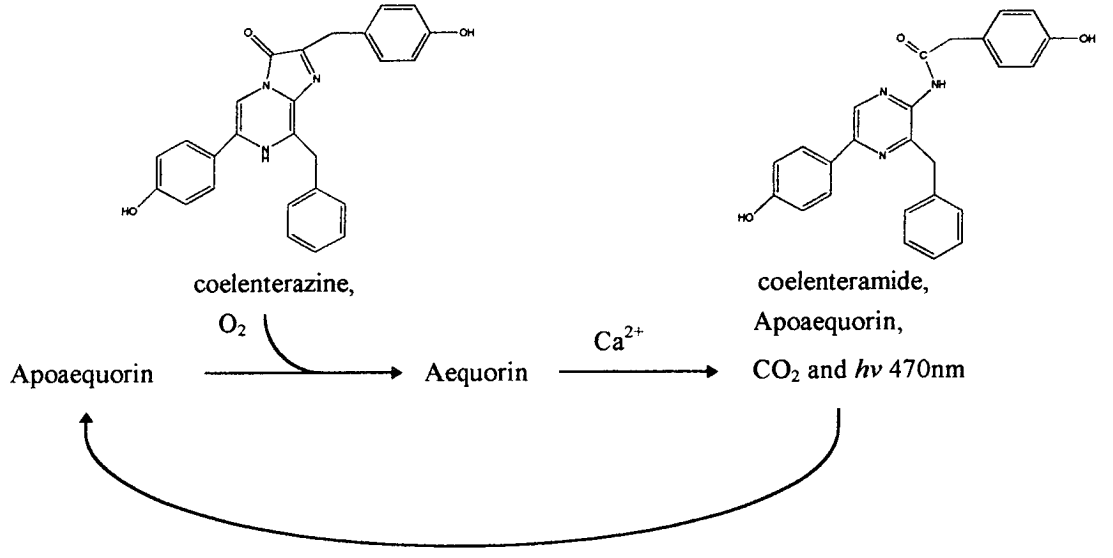
There are a wide range of Ca^{2+}-sensitive dyes, such as Fluo-3, Indo-1, and Fura-2 (Cobbold and Rink, 1987). They can be loaded into cells using various methods, including microinjection, ionophoresis or pressure injection. Some of these are ratioable, and therefore can be used to give quite accurate measurements of $[Ca^{2+}]_c$ (Read *et al.*, 1992; Read *et al.*, 1993; Parton and Read, 1999). With filamentous fungi however, injection methods are difficult due to the high turgor pressure within the cells. Also, in filamentous fungi the free dye is rapidly sequestered into organelles, preventing accurate measurement of $[Ca^{2+}]_c$ (Knight *et al.*, 1993b). Using pressure injection in *N. crassa*, this has recently been overcome by using 10 kDa dextran-dye conjugates to successfully measure cytosolic pH (Parton *et al.*, 1997) and $[Ca^{2+}]_c$ (Read *et al.*, 1998).

1.2.2 Aequorin

Aequorin is a Ca^{2+}-sensitive photoprotein naturally produced by the jellyfish' *Aequorea victoria* (Tsuji *et al.*, 1986; Cormier *et al.*, 1989). It was the first Ca^{2+}-sensitive photoprotein to be isolated (Shimomura *et al.*, 1962), and was immediately recognised as a possible indicator for low Ca^{2+} concentrations in biological systems (Shimomura *et al.*, 1963). It is composed of a 21.5 kDa apoprotein, apoaequorin, bound oxygen, and an organic substrate, coelenterazine. There are several isoforms of apoaequorin known (Prasher *et al.*, 1987), of which the complete cDNA has been isolated for two; apoaequorin a (*aeqA* or *aeq*1) (Prasher *et al.*, 1986), and apoaequorin d (*aeqD* or *AQ*440) (Inouye *et al.*, 1985). Upon binding calcium, aequorin emits light in a dose-dependent manner (Tsuji *et al.*, 1986; Cormier *et al.*, 1989), and therefore can be used to measure $[Ca^{2+}]_c$ in a cell (Figure 1.2).

The formation and reaction of aequorin is shown in Figure 1.2. Coelenterazine is a substituted dihydropyrazinimidazolone ring structure (M_r 423) and is a common substrate for marine bioluminescent reactions (Campbell and Herring, 1990). Molecular oxygen is thought to be bound to both the protein and coelenterazine. NMR studies have suggested that one of the oxygen atoms of the molecular oxygen is bound to the C-2 carbon of coelenterazine (Musicki *et al.*, 1986), with the other oxygen atom presumably providing a peroxide bridge to an amino acid of apoaequorin, which would stabilise the complex and prevent spontaneous luminescence.

Figure 1.2: Ca^{2+}-dependent luminescence of aequorin

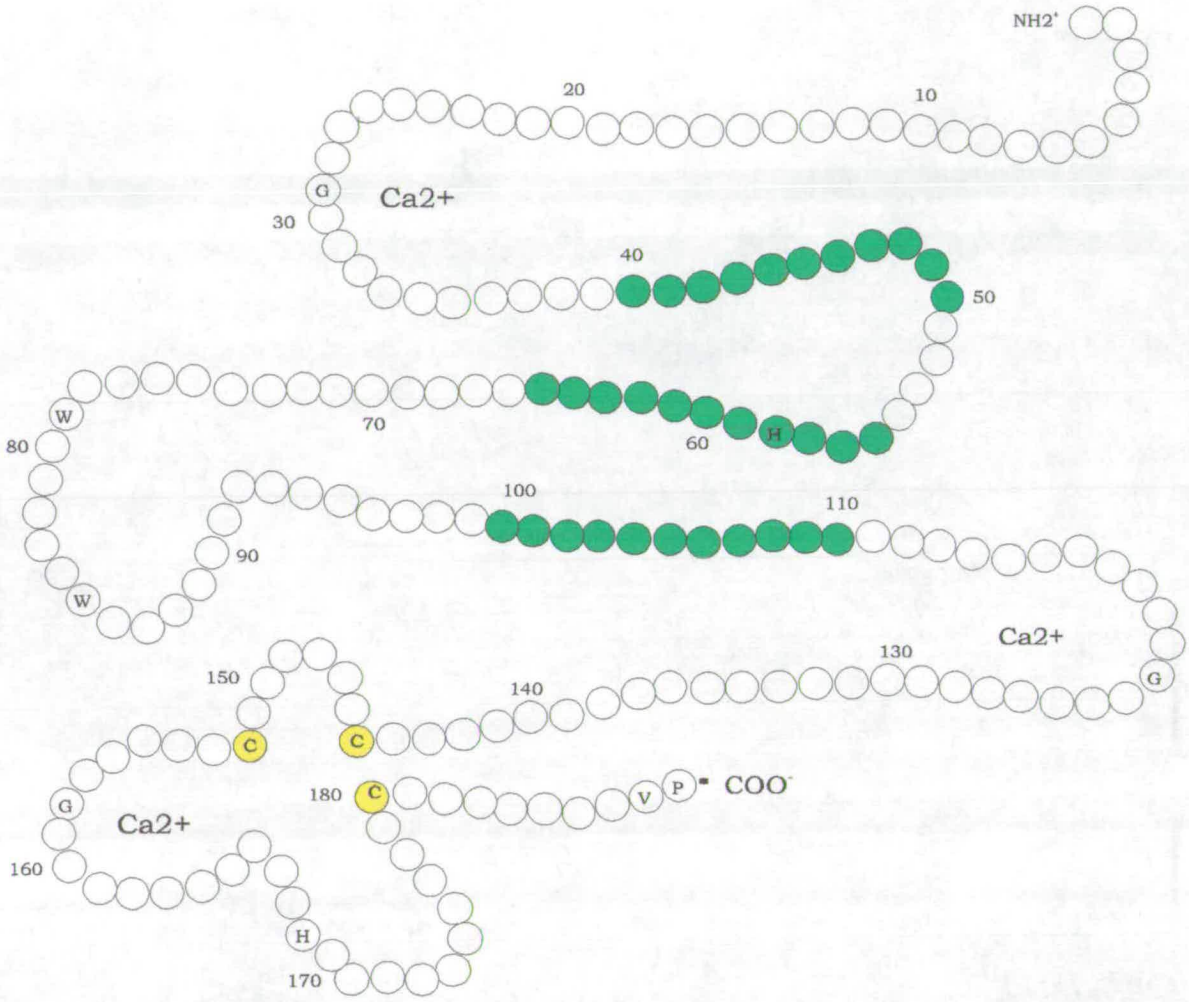


Key: Incorporation of Ca^{2+} and release of light is very fast (half rise time of approx. 6 ms). The formation of aequorin from apoaequorin, coelenterazine and O_2 is much slower. Chemical structures of coelenterazine and coelenteramide shown.

Upon binding Ca^{2+}, aequorin is converted to an intermediate compound (the blue fluorescent protein), which is a readily dissociable complex of aequorin and coelenteramide (Shimomura and Johnson, 1969; Shimomura and Johnson, 1973). The release of light in this reaction is known as chemiluminescence, because light energy is released only once as a result of a chemical reaction involving coelenterazine being converted into coelenteramide (the light emitter). This is distinct from fluorescence in which a fluorophore can emit light repeatedly after appropriate excitation. In its natural environment (in *A. victoria*), light is not released from aequorin because the energy of the reaction is instead transferred to green fluorescent protein (GFP), by free resonance energy transfer, which then emits green light (Tsuji *et al.*, 1986; Prasher, 1995). The energy released as luminescence in the absence of GFP is thought to be generated by the formation of a cyclic oxygen intermediate called dioxetanone. Evidence for the existence of this intermediate has been suggested by chemical synthesis of such a structure (Usami and Isobe, 1995).

Coelenterazine is non-cytotoxic, and being a very hydrophobic molecule is readily membrane-permeant. Also, there are many different coelenterazine analogues which have been chemically synthesised, and some of these can be used to alter the properties of active aequorin (Hirano *et al.*, 1995). Coelenterazine analogues have been used successfully for $[Ca^{2+}]_c$ measurement in transgenic *Nicotiana plumbaginifolia* expressing apoaequorin (Knight *et al.*, 1993a). However, the synthetic coelenterazine analogues can have drawbacks. One analogue, *e*-coelenterazine has been reported to have very poor cell permeability (Shimomura, 1997), although its use can allow ratiometric quantitation of $[Ca^{2+}]$ without knowledge of how much aequorin is present due to its bimodal luminescence (Shimomura, 1995). Most synthetic coelenterazine analogues also show lower stability, making them only suitable for measurement over a few hours (Shimomura *et al.*, 1993; Collis, 1996).

Figure 1.3: Primary structure of apoaequorin



Key: Primary structure shown as amino acid string. Ca^{2+} binding loops labelled (at amino acids (a.a.) 24-35, 117-128, and 152-164. Hydrophobic regions highlighted in green. Cysteine residues shown in yellow. Amino and carboxy terminals labelled. Labelled a.a. referred to in Table 1.2. Adapted from Tsuji, *et al.*, 1986.

Several attempts have been made to identify the essential sites for Ca^{2+} binding, coelenterazine binding and oxygen binding within apoaequorin. Since the isolation of cDNA for apoaequorin isoforms, this has been possible using site-directed mutagenesis (Table 1.2).

Table 1.2: Site-directed mutagenesis studies upon apoaequorin d

Amino acid (a.a)	Mutation	Luminescence (% wild type)	Reference
a.a. 1-14	Δ	0	Watkins & Campbell, 1993
Trp 12	Phe	13.2	Ohmiya *et al.*, 1992
His 16	Ala	25.7	Ohmiya & Tsuji, 1993
	Phe	0.1	Ohmiya & Tsuji, 1993
His 18	Ala	20.0	Ohmiya & Tsuji, 1993
His 27	Ala	69.8	Ohmiya & Tsuji, 1993
	Pro	67.2	Ohmiya & Tsuji, 1993
Gly 29	Arg	0	Tsuji, *et al.*, 1986
His 58	Phe	0-1.4	Ohmiya & Tsuji, 1993
			Tsuji *et al.*, 1986
	Tyr	14.9	Ohmiya & Tsuji, 1993
Trp 79	Phe	86 (124)	Ohmiya *et al.*, 1992
Trp 86	Phe	17.7 (bimodal) (45.6)	Ohmiya *et al.*, 1992
Trp 108	Phe	3.2	Ohmiya *et al.*, 1992
Gly 122	Arg	49	Tsuji *et al.*, 1986
Trp 129	Phe	24.6	Ohmiya *et al.*, 1992
Cys 145	Arg	48	Tsuji *et al.*, 1986
	Ser	61-67	Tsuji *et al.*, 1986
			(Kurose *et al.*, 1989)
Cys 152	Ser	46-59	Tsuji *et al.*, 1986
			Kurose *et al.*, 1989
Gly 158	Arg	97	Tsuji *et al.*, 1986
His 169	Phe	0.1	Ohmiya & Tsuji, 1993
	Ala	1.1	Ohmiya & Tsuji, 1993
	Trp	0	Ohmiya & Tsuji, 1993
Trp 173	Phe	0.6	Ohmiya *et al.*, 1992
Cys 180	Ser	13-29	Tsuji *et al.*, 1986
			Kurose *et al.*, 1989
Cys 145 +152	2 xSer	1	Kurose *et al.*, 1989
	n/a	disulphide bond	(Ohmiya *et al.*, 1993)
all 3 Cys	3 xSer	21 [116]	Kurose *et al.*, 1989

Table 1.2 continued.

Amino acid (a.a)	Mutation	Luminescence (% wild type)	Reference
Val 188	Δ	<0.1	(Nomura *et al.*, 1991)
Pro 189	Δ	0	Nomura *et al.*, 1991
			(Watkins and Campbell, 1993)
a.a 187-189	Δ	0.02	Nomura *et al.*, 1991
+Gly (190)	C-term. add^{n}	1.4	Nomura *et al.*, 1991

Key: relative luminescence levels given as percentage compared to native aequorin luminescence (100%). Relative luminescence levels in parentheses are obtained with semi-synthetic aequorin reconstituted with *e*-coelenterazine, and are relative to semi-synthetic native aequorin with the same coelenterazine analogue. Luminescence level in square brackets represents luminescence obtained after a 3 times longer aequorin generation time, compared to native aequorin with the same generation time.

Apoaequorin cDNA was isolated by Inouye *et al.* (1985) and the complete apoaequorin peptide is 196 amino acids (Figure 1.3).

Apoaequorin contains three Ca^{2+}-binding domains (EF hands, Figure 1.3), which show strong homology both in amino acid sequence and position to three of the four EF hands present in CaM and troponin C. This suggests a common evolutionary origin for the proteins (Charbonneau *et al.*, 1985). Aequorin shows a strong affinity for Ca^{2+} (K_D = 0.14 μM), but other ions will also bind in the EF hands. As a result, Sr^{2+}, Pb^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+} and lanthanides will all trigger aequorin luminescence, some other ions will competitively inhibit (Na^{+}, K^{+} and Mg^{2+}), and others will inhibit non-competitively (Ag^{2+} and Hg^{2+}), all to different extents depending upon their ability to bind to aequorin (Shimomura *et al.*, 1962; Shimomura and Johnson, 1969). Of these ions, only Mg^{2+} and K^{+} are present in biological systems at levels which may affect aequorin luminescence. Since Mg^{2+} and K^{+} both competitively inhibit Ca^{2+} binding to aequorin, they can both be expected to desensitise aequorin to small changes in $[Ca^{2+}]$. However, *in vitro* luminescence of aequorin under conditions that mimic physiological conditions show that aequorin can be used to accurately measure Ca^{2+} over a large range of $[Ca^{2+}]$ (Shimomura, 1991). pCa represents molar Ca^{2+} concentration as $-\log_{10}[Ca^{2+}]$, and 5.2-6.7 covers the physiological range of Ca^{2+}

concentrations (approx. 10^{-5}-10^{-7} M Ca^{2+}). This suggests that the levels of Mg^{2+} and K^{+} present within cells are insufficient to greatly affect accurate measurement of $[Ca^{2+}]_c$ with aequorin.

Comparing the results obtained using site-directed mutagenesis with the primary structure of apoaequorin (Figure 1.3) shows that only the first two Ca^{2+} binding domains are required for luminescence (Tsuji *et al.*, 1986); replacement of the essential Gly residue of the third Ca^{2+} binding domain (Gly 158) with Arg (positively charged a.a.) producing no effect upon relative luminescence. This observation is validated by luminometric titration of homogenous recombinant aequorin showing a requirement for two Ca^{2+} ions for luminescence (Shimomura and Inouye, 1996). The affinity of binding of the first two Ca^{2+} ions is approximately (approx.) 22 times greater than that for the third. Deletion of the carboxy terminal Pro (a hydrophobic a.a.) abolishes luminescence activity, as does adding a non hydrophobic a.a. onto the carboxy terminus (Nomura *et al.*, 1991). It has been suggested that Pro 189 is required for the long term stability of aequorin, and that it plays a role in securing coelenterazine to apoaequorin (Watkins and Campbell, 1993). Mutagenesis of either His 58 or His 169 to Phe abolishes activity, and both have been suggested as the amino acid that binds oxygen to form the peroxide bridge to coelenterazine (Tsuji *et al.*, 1986; Ohmiya and Tsuji, 1993). Replacement of Trp 86 with Phe changes the luminescence to a dual emission spectrum, suggesting that Trp 86 plays a part in producing the excited state of coelenteramide (Ohmiya *et al.*, 1992). Evidence from site-directed mutagenesis and tryptic digestion of apoaequorin suggest that a disulphide bridge exists between Cys 145 and Cys 152 (Kurose *et al.*, 1989; Ohmiya *et al.*, 1993). The presence of a disulphide bridge has been shown to decrease the aequorin generation time (Ohmiya *et al.*, 1993).

In addition to the mutagenesis studies, several fusion proteins have been constructed, fusing another gene upstream of the apoaequorin gene. This produces an N-terminal fusion to apoaequorin, and all reported fusion proteins have shown aequorin activity

(Casadei *et al.*, 1990; Zenno and Inouye, 1990; Brini *et al.*, 1993). This suggests that the N-terminal of aequorin does not play a part in co-ordinating the components of the active site.

Overall, the structure/function relationship of aequorin is not very clear, except for the Ca^{2+}-binding which bears striking similarity with CaM. Almost all mutations of the amino acid sequence result in decreased luminescence. Attempts to discover the exact binding site of both oxygen and coelenterazine have not produced conclusive answers. N-terminal fusion proteins produce active aequorin which suggests that the there is a degree of flexibility in the N-terminal region of the protein sequence, whereas modification of the C-terminus drastically inhibits luminescence.

1.2.2.1 Detection of aequorin luminescence

The properties of aequorin mean that a very sensitive detection system is required to accurately measure luminescence. The production of light is very rapid, with a half rise time of ~6 ms (Hastings *et al.*, 1969), and the quantum yield is low, between 0.15 and 0.23 at 25 °C (Cobbold and Rink, 1987; Shimomura and Johnson, 1970). Photomultiplier tubes allow rapid amplification of very small signals by generating an electron for every photon detected at the photocathode light detector. Each electron then passes down a series of dynodes, being amplified at each dynode, to finally output a pulse of electrons at the anode. This occurs very quickly (<100 ns), and the pulse is then delivered to a discriminator which selects charge above a certain energy level to remove background noise. The output is then sent to a suitable recorder/data accumulator. The ability to detect down to one photon and accurately measure over a large dynamic range makes the photomultiplier tube an excellent detector for aequorin chemiluminescence. Instruments this sensitive can detect aequorin as low as 1×10^{-18} g.

Spatial resolution of aequorin luminescence from cells can be achieved by imaging using a low light camera (*e.g.* a photon counting camera, intensified tube or a

charged coupled device (CCD) camera; Campbell, 1988). CCD cameras rely upon a two dimensional array of semiconductors, each of which picks up light from a certain area of the sample. The pattern of released light picked up by the semiconductors can then be constructed as a two dimensional image using a computer and compared to the bright field image of the sample. This has been used to measure successfully bioluminescence in *Dictyostelium* (Cubitt *et al.*, 1995) and in transgenic *N. plumbaginifolia* plants expressing apoaequorin from *aeqA* under the control of the 35S promoter (Knight *et al.*, 1993a).

In summary, aequorin has a number of advantages for measuring $[Ca^{2+}]_c$. The ability to transform organisms with apoaequorin genes and express the apoprotein without disrupting the cell allows simple generation of aequorin by incubating cells in coelenterazine. This is non-invasive compared to methods such as pressure injection, which is required to measure $[Ca^{2+}]_c$ using fluorescent dyes in filamentous fungi. Also, aequorin can measure Ca^{2+} over a large range of concentrations, exhibits very little Ca^{2+} buffering and is non cytotoxic. The major disadvantage of aequorin is the low quantum yield of light emitted, making it difficult to ensure enough light is produced to measure basal $[Ca^{2+}]_c$, which is typically very low. Overcoming this problem requires producing apoaequorin to a high level in the system of interest. The obvious method for obtaining high levels of aequorin which would allow long term measurement of $[Ca^{2+}]_c$ is to transform the desired organism with an apoaequorin gene under the control of a strong promoter.

1.3 Gene expression in filamentous fungi

Fungi secrete a diverse selection of primary and secondary metabolites (*e.g.* organic acids and antibiotics). For this reason, fungi have been studied for many years for their use in industrial processes. Classical genetic studies have revealed a lot about their genetics and biochemical pathways. The development of molecular biology

techniques have allowed this to be taken further, and have allowed the characteristics of filamentous fungi to be taken advantage of to produce high levels of heterologous proteins. Heterologous protein production has been carried out in several species of *Aspergilli*, including *A. nidulans*, *A. niger*, *A. awamori* and *A. oryzae*. Gene expression studies in *Aspergillus* are carried out to try and improve strains for industrial fermentation to yield higher levels of heterologous proteins (Finkelstein, 1991; May, 1992).

Table 1.3: Examples of successful production of heterologous proteins in *Aspergillus* spp.

Protein produced	Host species	Reference
Bovine prochymosin	*A. niger*	(van Hartingsveldt *et al.*, 1990)
	A. awamori	(Dunn-Coleman *et al.*, 1991; Ward *et al.*, 1990)
	A. nidulans	(Cullen *et al.*, 1987)
	A. oryzae	(Tsuchiya *et al.*, 1994)
Hen Egg White Lysozyme	*A. niger*	(Jeenes *et al.*, 1993; Archer *et al.*, 1990)
Human Interleukin-6	*A. nidulans*	(Contreras *et al.*, 1991; Carrez *et al.*, 1990)
	A. niger	(Broekhuijsen *et al.*, 1993)
Human Lactoferrin	*A. nidulans*	(Ward *et al.*, 1992a)
	A. oryzae	(Ward *et al.*, 1992b)
	A. awamori	(Ward *et al.*, 1995)
Porcine Pancreatic Prophospholipase A_2	*A. niger*	(Roberts *et al.*, 1992)

Species other than *Aspergillus* have also been used for heterologous protein production, such as *Trichoderma reesei* for the production of mammalian antibody fragments (Nyyssönen *et al.*, 1993; Nyyssönen and Keränen, 1995). However, *Aspergilli* have been used far more than other species, as shown by the number of heterologous proteins produced using such strains (Table 1.3).

1.3.1 Heterologous gene expression

Obtaining high levels of heterologous protein production in filamentous fungi requires a transformation procedure with a relatively high success rate, so that enough transformants can be analysed for their protein production levels. Large differences in protein production are seen between transformants because of the method of transformation. Unlike yeast and prokaryotes, in which expression vectors can be maintained autonomously, fungi generally require integration into the genome for stable transformation. This introduces variation in integration site and copy number between transformants, which produces different levels of protein in different transformants. Attempts to circumvent this variation have led to the development of autonomously replicating vectors which do not integrate into the genome (Gems *et al.*, 1991; Verdoes *et al.*, 1994b). The transformation rate with such vectors is reported to be 10-100 fold greater than that with integrative plasmids (Verdoes *et al.*, 1994b). However, the resultant transformants are mitotically unstable, making the transformants only suitable for short term analysis.

1.3.1.1 Transformant selection

Several methods of transformation have been developed for fungi using either complementation of auxotrophic mutants with the wild type gene or dominant selectable markers (reviewed by Ballance, 1991). The transformation rates obtained vary depending upon the strain used and whether single or double selection is used. Also, the type of transformants obtained can depend upon the selection marker used. Transformed DNA may integrate homologously at a site containing DNA sequence identical to part of the vector, or by gene replacement inserting only the identical part of the vector at the homologous site. The other alternative is ectopic integration, apparently at random within the genome, which generally inserts a variable number of copies of the transforming vector in tandem arrays.

Homologous integration or gene replacement can occur with homologous auxotrophic selection markers for which there is a mutant copy present in the genome. Examples of auxotrophic selection markers are selection of nitrate reductase (*niaD*) mutants on chlorate (Gouka *et al.*, 1991), acetyl-coenzyme A synthetase (*facA*) mutants on fluoroacetate (Gouka *et al.*, 1993) and orotidine-5'-monophosphate-decarboxylase (designated *pyrG*, *pyrA*, *pyr4*, or *ura3*) mutants on 5-fluoro-orotic acid (Goossen *et al.*, 1987; van Hartingsveldt *et al.*, 1987). Both forms of integration are site-specific, but gene replacement loses the non-homologous parts of the vector and only inserts one copy of the selection marker. Homologous integration generally only inserts one copy of the plasmid at the site of the mutated selection gene. This allows insertion of other genes within the plasmid at a known part of the genome, which may avoid the problems of gene silencing at ectopic integration sites. An alternative method of selection to direct homologous recombination is to use a mutated auxotrophic marker, which is selected for only if it integrates at the site of the wild type gene within the genome. Such a method has been employed using the *pyrG* gene of *A. niger* (van Gorcom and van den Hondel, 1988). Transformation of a wild type *pyrG* strain with a vector containing a mutated form of *pyrG* dramatically increases the rate of homologous recombination compared to ectopic recombination, but with a much lower rate of transformation (van Gorcom and van den Hondel, 1988).

Ectopic integration is more common when using vectors containing non-homologous DNA, such as dominant selection markers. The major disadvantages of ectopic integration is that integration is random, which may result in the disruption of essential genes, or that the transforming DNA may integrate at a silent region of the genome. Selection will rule out integration in silent parts of the genome but cannot determine the effects of ectopic integration in successful transformants, which may disturb the original phenotype. The advantages of ectopic integration are that it can be used to transform wild type strains if they are sensitive to the selection procedure. Also, integration generally inserts tandem arrays of several copies of the vector,

which helps increase the level of expression of the transformed genes. Examples of such markers are hygromycin resistance (Punt *et al.*, 1987; Punt and van den Hondel, 1993) and use of the *A. nidulans* acetamidase gene (*amdS*) which allows growth on acetamide or acrylamide as a nitrogen source in species lacking any such gene, such as *A, niger* and *A. awamori* (Kelly and Hynes, 1985). In respect of selecting high expression levels of the transformed genes, selection of *amdS* using acrylamide as a nitrogen source requires higher amounts of acetamidase than using acetamide, which is a useful means of selecting only high expression transformants (Kelly and Hynes, 1985).

In several cases, multicopy transformants obtained by integration of tandem repeats have been shown to be mitotically stable after several rounds of serial isolation (Kelly and Hynes, 1985; Wernars *et al.*, 1985; Cullen *et al.*, 1987). Generally, protein yield increases with copy number, although it may not be strictly proportional, as some ectopic sites of integration will be transcribed more than others (Baron *et al.*, 1992). Also, integration of excessive copy numbers may decrease expression due to titration of trans-acting regulatory proteins which bind to the promoter (Verdoes *et al.*, 1994a).

1.3.1.2 Expression vectors

Successful transformation, and subsequent high expression of the transformed gene requires several factors to be considered in constructing the expression plasmid (reviewed by van den Hondel *et al.*, 1991). The expression cassette employed must have a suitable transcriptional control region upstream of the gene to be expressed. Also, a suitable terminator that is known to be functional in the transformed species is required for production of stable mRNA. If the gene product is to be targetted to a specific compartment, such as the ER or vacuole, or to be secreted, then a signal sequence is also required for successful processing of the translated product. To maximise the level of expression, the expression signals (*i.e.* the promoter and terminator) should be native to the host species and also from a highly expressed

gene. However, the transcriptional control and translation initiation regions contained within a promoter from one fungal species may function successfully in a wide range of related fungal species (van den Hondel *et al.*, 1991). The role of the terminator in the expression cassette is not fully understood. This region possesses the sequence for transcription termination and encodes the polyadenylation signal, both of which are thought to be required for optimal mRNA production and stability.

Suitable vectors for expression in *A. niger* and *A. awamori* have been produced by workers at TNO, Zeist, The Netherlands (van den Hondel *et al.*, 1991). These vectors contain suitable promoter and terminator regions around unique restriction sites for incorporation of the gene to be expressed. The vectors also contain suitable sequences to allow their use as shuttle vectors for manipulation in *E. coli* (*i.e.* they contain a bacterial origin of replication and selection marker). Fungal selection markers can be incorporated using other restriction sites within the vector, or alternatively, a separate selection vector may be used in co-transformation. Expression vectors with both inducible and constitutive promoters have been produced, both with and without signal sequences for export of the translated product.

pAN52-1 is a vector designed at TNO which contains the required sequences for use as a shuttle vector and fungal expression vector (van den Hondel *et al.*, 1991). The promoter employed is the glyceraldeyhyde-3-phosphate dehydrogenase (*gpdA*) promoter from *A. nidulans* and the terminator region is from the *trpC* gene of *A. nidulans* (Mullaney *et al.*, 1985). The promoter is constitutive, and both sequences are known to be active in *Aspergillus niger* (Punt *et al.*, 1987). A short sequence between the promoter and the terminator regions contains two unique restriction sites, one of which (*Nco*I) cuts across the ATG start codon, allowing insertion of a gene in the same start position as would *gpdA* in *A. nidulans*. The other (*Bam*HI) allows correct orientation of the inserted gene if the gene is engineered to have a suitable restriction site 3' of the coding sequence. A third

unique restriction site (*Eco*RI) exists upstream of the *gpdA* promoter, which may be used to insert a selectable marker expression cassette. The remaining backbone of the vector is derived from a bacterial plasmid and contains a bacterial origin of replication and ampicillin resistance for selection in *E. coli*.

1.3.1.3 Production of heterologous protein

Production of intracellular proteins in filamentous fungi has been studied less than that of extracellular production, as the latter is of more use commercially. In *Aspergillus*, Punt *et al.*, (1991) showed that production levels of the *E. coli* enzymes β-galactosidase and β–glucouronidase were in the order of that seen for a constitutive homologous protein, glyceraldehyde-3-phosphate dehydrogenase of *A. nidulans*.

Production of extracellular heterologous proteins is usually much lower than that obtained for homologous fungal proteins (reviewed by van den Hondel *et al.*, 1991), although the genes are usually transcribed as efficiently as fungal genes. This suggests that problems occur post-transcription, due to poor translation, degradation, or incorrect/inefficient processing.

Intracellular production of the heterologous protein, aequorin (*aeqA*), in *N. crassa* has been found to be limited post-transcriptionally (Collis, 1996). The protein's expression level was very low (approx. 10 ng of active aequorin per g total soluble protein). This is undetectable on a Western blot, and could only be calculated through measurement of the bioluminescence of aequorin. A reasonable signal for *aeqA* mRNA was obtained, as shown through Northern blots. Therefore, it appeared that there was a block at the translation stage or later, in apoaequorin synthesis. The probable causes suggested for this were either protein instability or codon usage. The former was discounted, as aequorin stability in whole cell extract was monitored and shown to be reasonable (half life approx. 45 min). Therefore, codon bias was

found to be the most likely explanation for very low translation of apoaequorin in *N. crassa* (Collis, 1996).

Differential use of codons between species is a possible explanation for the low levels of protein produced using homologous-heterologous gene fusion constructs. Comparison of the gene fusion protein levels with those of the homologous gene alone generally show lower levels for the fusion product. In *Trichoderma reesei*, yield from a cellobiohydrolase I - heavy Fd chain antibody fragment was found to be 150 mg/l compared to 1-10 g/l of cellobiohydrolase I on its own (Nyyssönen *et al.*, 1993). However, yields of the fusion protein were far higher than that obtained from the heterologous protein alone (Nyyssönen and Keränen, 1995). Similar results were obtained with a hen egg white lysozyme-glucoamylase fusion gene in *A. niger*, where a ten-fold higher level of mRNA : protein ratio was obtained for the homologous gene alone compared to the fusion gene (Jeenes *et al.*, 1994).

1.3.2 Codon usage

Codon bias can occur for any amino acid that has two or more synonymous codons. Originally described in *E. coli* (Ikemura, 1981), the choice of codon was shown to be correlated with the abundance of the corresponding tRNA. Highly expressed genes showed the greatest bias, with little or no bias in low level expression genes. Similar findings have been shown in *S. cerevisiae* (Ikemura, 1985). Codon usage tables have since been produced for many organisms, based upon genetic databases such as Genbank (Nakamura *et al.*, 1998). Analysis of codon usage in *N. crassa* (Edelmann and Staben, 1994) and *A. nidulans* (Lloyd and Sharp, 1991) have shown that both fungi prefer pyrimidines in the third position of the codon, and when a purine is obligatory, they prefer guanine over adenine. Introduction of three rare codons into the glutamate dehydrogenase gene of *N. crassa* caused a 70% loss in protein production without affecting the mRNA level (Kinnaird *et al.*, 1991), highlighting the importance of optimal codon usage for high expression. It has been suggested

that the presence of rare codons hinders translation, causing ribosome pausing (or stalling), which effectively causes a 'traffic jam'. This is enhanced by having several rare codons in a row (Gurski *et al.*, 1992).

Since each species has different codon usage, expression of a heterologous gene may be inhibited by inappropriate choice of codons within the sequence. To avoid such possibilities, several synthetic genes have been produced which choose the optimal codons for the expression host to be used. These studies have yielded mixed results. A re-engineered GFP gene sequence which favoured codons of highly expressed human proteins gave 20-fold higher GFP expression in *Zea mays* cells than the original jellyfish gene (Chiu *et al.*, 1996). Expression of native GFP in *C. albicans* produces no detectable fluorescence. In comparison, optimizing the gene for *C. albicans* (called yeast enhanced GFP, [yEGFP]) produced high levels of expression (Cormack *et al.*, 1997). In *S. cerevisiae* no change in expression of the murine interleukin-2 gene was observed after replacement of twelve consecutive codons with those optimal for the host (Demolder *et al.*, 1992). In *Streptomyces lividans* replacing certain rare codons in the mouse tumour necrosis factor α gene did not improve yield, and when five of these were adjacent a drastic reduction in protein level was observed due to a marked decrease in mRNA stability (Lammertyn *et al.*, 1996). By expressing codon-adjusted synthetic α and β-globin genes in *E. coli*, a high protein yield was obtained but only when the two genes were expressed as a fusion protein (Hernan *et al.*, 1992).

Altering codon usage does not appear to be completely straightforward, with other factors having to be taken into account, such as mRNA stability and the possible presence of cryptic sequences within the heterologous gene. The extent of influence that codon usage exerts upon protein levels is still not completely known. However, it is a possible method for increasing heterologous gene expression. Combined with the findings obtained previously by Collis (1996), this was considered an option worth exploring for increasing aequorin expression in filamentous fungi.

1.4 Outline of thesis

There is no method currently used for routinely measuring $[Ca^{2+}]_c$ in filamentous fungi. One possible method is to use recombinant aequorin. The findings of Collis (1996), suggested there were limitations on aequorin production in *N. crassa* due to codon bias. In this thesis, this hypothesis is tested. The aims of this study were:

- To produce a synthetic apoaequorin gene that utilised the preferential codons of *Aspergillus* and *Neurospora*. This is the first time that this has been done with apoaequorin.

- To compare the expression levels obtained with the synthetic gene with those obtained with the native apoaequorin gene.

- To increase expression levels of apoaequorin in filamentous fungi using appropriate expression vectors and hosts for heterologous gene expression. Two species of *Aspergillus*; *A. niger* and *A. awamori* were chosen for transformation, due to the availability of successful transformation procedures, tried and tested high expression vectors and the abundant knowledge regarding heterologous gene expression in these organisms. This is the first time aequorin has been expressed in *Aspergillus*.

- To evaluate the recombinant aequorin method for measuring $[Ca^{2+}]_c$ in filamentous fungi, and then to use a transformant expressing apoaequorin to a high level to investigate the effects of various stimuli upon $[Ca^{2+}]_c$. This work demonstrated for the first time that recombinant aequorin can be used to routinely measure $[Ca^{2+}]_c$ in filamentous fungi.

2. MATERIALS AND METHODS

2.1 Chemicals

Unless otherwise specified, media reagents were purchased from BDH Chemicals Ltd (UK) and biochemicals from Sigma (UK). Restriction endonucleases, modification enzymes, isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) were obtained from Boehringer Mannheim (UK). Hybond-N nylon membrane and Hybond-C Extra nitro-cellulose discs (132 mm) were purchased from Amersham International plc. (UK). Synthetic oligonucleotides were purchased from Oswel DNA Service (UK) or made in-house at Zeneca Agrochemicals (Jealott's Hill, Berkshire, UK). The oligonucleotides used in this study are shown below in Table 2.1and Table 2.2.

Table 2.1: PCR and sequencing primers

AeqDA -primer A, 20 bases, +strand	CCGCAGACCT GAATTCATGA
AeqDB -primer B, 30 bases, -strand	TTTTGGATCC CTTGGCGTAC TTCTCGAGCT
AeqDC -primer C, 30 bases, +strand	TTTTGAATTC GCCACCGACG AGCTCGAGAA
AeqDD -primer D, 20 bases, -strand	TCAGGCTGGA TCCAAGCTTA
AeqDE -primer E, 20 bases, -strand	TACTTCTCGA GCTCGTCGGT
AeqDF -primer F, 20 bases, +strand	ACCGACGAGC TCGAGAAGTA
AeqDFI -primer, 21 bases, +strand	CGAGGACTGC GAAGAGACCT T
AeqDRI -primer, 21bases, -strand	AAGGTCTCTT CGCAGTCCTC G
AeqDFII -primer, 20 bases, +strand	TCCGACATCG TCATCAACAA
AeqDRII -primer, 20 bases, -strand	TTGTTGATGA CGATGTCGGA
pTAg SEQ 5' primer, 22 bases, +strand	GCTATGACCA TGATTACGCC AA
pTAg SEQ 3' primer, 21 bases, -strand	TGTAAAACGA CGGCCAGTGA A
T7 promoter, 20 bases, +strand	TAATACGACT CACTATAGGG
SP6 promoter, 19 bases, -strand	TATTTAGGTG ACACTATAG

Table 2.2: Synthetic oligonucleotides covering Apoaequorin D gene

AeqD1: +strand	CCGCAGACCT GAATTCATGA CCTCCAAGCA GTACTCCGTC AAGCTTACCT CCGACTTCGA CAACCCCCGC TGGATCGGCC GCCACAAGCA CATGTTCAAC TTCCTC
AeqD2: -strand	CGAGGTTGTT GATGACGATG TCGGAGGCCT TGTAGACCAT CTCGTCGAGG GAAATCTTGC CGTTGTGGTT GACGTCGAGG AAGTTGAACA TGTGCTTGTG GCGGCC
AeqD3: +strand	AGGCCTCCGA CATCGTCATC AACAACCTCG GCGCTACCCC CGAGCAGGCC AAGCGCCACA AGGACGCCGT CGAGGCCTTC TTCGGCGGTG CCGGCATGAA GTACGG
AeqD4: -strand	CTTGGCGTAC TTCTCGAGCT CGTCGGTGGC GAGCTTCTTC CAGCCCTCGA TGTAGGCGGG CCAGTCGGTC TCGACGCCGT ACTTCATGCC GGCACCGCCG AAGAAG
AeqD5: +strand	GCCACCGACG AGCTCGAGAA GTACGCCAAG AACGAGCCCA CCCTCATCCG CATCTGGGGC GACGCCCTCT TCGACATCGT CGACAAGGAC CAGAACGGTG CCATCA
AeqD6: -strand	CGGAAGGTCT CTTCGCAGTC CTCGCTGGAC TGAATGATGC CGGCGGCCTT GGTGTAGGCC TTCCACTCGT CGAGGGTGAT GGCACCGTTC TGGTCCTTGT CGACGA
AeqD7: +strand	GTCCAGCGAG GACTGCGAAG AGACCTTCCG CGTCTGCGAC ATCGACGAGT CCGGCCAGCT CGATGTCGAT GAGATGACCC GCCAGCACCT CGGCTTCTGG TACACC
AeqD8: -strand	TCAGGCTGGA TCCAAGCTTA GATCTTAGGG GACGGCACCG CCGTAGAGCT TCTCGCAGGC GGGGTCCATG GTGTACCAGA AGCCGAGGTG CTGGCGGGT

Key for tables 2.1 and 2.2: All oligonucleotides are written 5'-3'. Oligos in table 2.2 all overlap by 30bp.. For use of oligonucleotides and primers, see section 2.7. For uses of primers in sequencing, see section 2.8

Table 2.3: Plasmids and their selective genes

plasmid	**uses**	**selection genes**	**Supplier/ reference**
pTAg	PCR T cloning vector	*amp*^{R}, *kan*^{R}, *lacZ*	R&D Systems, UK
pGEM3z	general cloning vector	*amp*^{R}, *lacZ*	Promega, UK
pET16b	His-tag expression vector	*amp*^{R}	Novagen, UK
PNOM102	fungal expression vector	*amp*^{R}	(Punt *et al.*, 1991)
pAW4155S	fungal selection vector	*amp*^{R}, *amdS*	(Wernars *et al.*, 1985)
pAB4-1	fungal selection vector	*pyrG*	(van Hartingsveldt *et al.*, 1987)

The plasmids used for DNA manipulation and expression of genes are given in Table 2.3.

2.2 Organisms and Media

Manipulation of organisms was carried out using established sterile technique. Genetically modified *N. crassa*, *A. niger*, *A. awamori* and *E. coli* were all containment level 1 organisms and the correct procedures for handling and disposal were followed (Genetic Manipulation and Biological Safety Committee, University of Edinburgh). All media and salt solutions were made up in distilled H_2O (dH_2O) and sterilized by autoclaving at 15 pounds per square inch for 20 minutes (min) prior to use. Heat labile components were filter sterilized individually and added to the main solution after the latter was autoclaved. Percentage solutions are weight : volume (w/v) unless stated otherwise.

2.2.1 *Aspergillus* strains

Two *Aspergillus* species were used for transformation; *A. niger* and *A. awamori*. The strains of each are shown in Table 2.4. All strains were obtained from TNO, The Netherlands.

Table 2.4: Strains of *Aspergillus* used for transformation

Strain	phenotype	reference
A. niger		
AB4.1	Δ*pyrG*⁻ (uridine auxotroph)	(van Hartingsveldt *et al.*, 1987)
AB1.13	Δ*pyrG*⁻ (uridine auxotroph) and protease deficient mutant of AB4.1	(Mattern *et al.*, 1992)
A. awamori		
CBS115.52 T#3	Δ*pyrG*⁻ uridine auxotroph of CBS115.52 (ATCC 11358)	supplied by J.G.M. Hessing, TNO (Gouka *et al.*, 1995)

2.2.1.1 Culture media

Aspergillus cultures were grown in rich media or minimal media with glucose as a carbon source. The media are based upon *Aspergillus* minimal media (Bennet and Lasure, 1991), and are given below in sections 2.2.1.1.1, 2.2.1.1.2, and 2.2.1.1.3. For solid media, 2% w/v Oxoid agar number 3 (Oxoid, UK) was added prior to

sterilisation. For growth of *pyrG*⁻ strains, all media were supplemented with uridine to a final concentration of 10 mM. Salt solutions for *Aspergillus* media are given in Table 2.5 below. For selection using a specific nitrogen source, the salt solution AspA-N was used, with the correct nitrogen salt being added separately.

Table 2.5: Salt solutions for *Aspergillus* media

	AspA	AspA-N
$NaNO_3$	150 g	--
KCl	13 g	13 g
KH_2PO_4	38 g	38 g
10 N KOH	<25 ml until pH 5.5	<25 ml until pH 5.5
dH_2O	to 500 ml	to 500 ml

Trace elements were added to the media from a stock solution, shown in Table 2.6.

Table 2.6: 1000x Trace elements for *Aspergillus* media

d H_2O	80 ml
$ZnSO_4.7\ H_2O$	2.2 g
H_3BO_3	1.1 g
$MnCl_2.4\ H_2O$	0.5 g
$FeSO_4.7\ H_2O$	0.5 g
$CoCl_2.6\ H_2O$	0.17 g
$CuSO_4.5\ H_2O$	0.16 g
$Na_2MoO_4.2\ H_2O$	0.15 g
EDTA	5 g

Compounds were added one by one in the order given in Table 2.6 until dissolved. The solution was then boiled and allowed to cool down to 60 °C. The pH was adjusted to 6.5 with concentrated KOH, then cooled down to room temperature and adjusted to 100 ml with dH_2O. The solution was sterilized for 20 min at 121 °C.

2.2.1.1.1 Minimal Media (MM) for *Aspergillus*

Table 2.7: Minimal Media (MM) for *Aspergillus*

	MM	MM-N
50x AspA-N (see Table 2.5)	-	10 ml
50x AspA (see Table 2.5)	10 ml	-
1 M $MgSO_4$	1 ml	1 ml
1000x trace elements (see Table 2.6)	0.5 ml	0.5 ml
50% glucose	10 ml	10 ml
dH_2O	to 500 ml	to 500 ml

2.2.1.1.2 Media for expression controlled by the *gpdA* promoter

Media was produced as described for MM (Table 2.7), with the addition of 2.5 g yeast extract prior to sterilization. 0.5 g casein amino acids were then added to the media.

2.2.1.1.3 Rich media with glucose (RMG)

This medium was used to produce a large amount of spores for inoculation of liquid media and long term storage of strains on silica gel (see section 2.2.1.2.2).

1 g neopeptone (trypticase BBL) and 0.5 g yeast extract were added to 450 ml dH_2O and sterilized for 20 min at 121 °C. Then following sterile components were then added:

50x AspA (see Table 2.5)	10 ml
1 M $MgSO_4$	1 ml
1000x trace elements (see Table 2.6)	0.5 ml
50% glucose	10 ml
10% casein amino acids	5 ml
1 M uridine for PyrG$^-$ strains	5 ml
dH_2O	to 500 ml

2.2.1.1.4 Selection media for *amdS* and *pyrG*$^+$ transformants

This media was used for selection of protoplasts that were co-transformed with the *Aspergillus nidulans amdS* gene and the *A. niger pyrG* gene (section 2.10.2).

To 450 ml dH_2O, 10 g Oxoid agar and 109.3 g sorbitol were added. The solution was sterilized for 20 min at 121 °C. The media was then made up as per MM-N (Table 2.7), with the addition of 5 ml 1.5 M CsCl and 5 ml 1 M acrylamide or 5 ml 1 M acetamide.

For serial isolation of transformants (section 2.10.3), the osmotic protectant (sorbitol) was not required and therefore omitted.

2.2.1.2 Culturing *Aspergillus*

2.2.1.2.1 Inoculation procedure

Unless otherwise specified, solid cultures were inoculated using a sterile platinum wire loop. For liquid media inoculation and short term storage, conidia were harvested from RMG plates (section 2.2.1.1.3), and stored in 0.8% NaCl (PZ) at 4 °C, as described in section 2.2.1.2.2. Known quantities of spores could then be aliquoted by pipette into the media.

2.2.1.2.2 Spore suspensions and silica gel stocks

A spore plate (see section 2.2.1.2.3.1) was harvested by gently adding 10 ml PZ and disrupting the conidia from the conidiophores with a sterile glass rod. The conidial suspension was poured into a sterile 14 ml round bottomed Falcon® tube (Becton Dickinson, New Jersey, USA), and the plate washed with a further 5 ml PZ which was added to the Falcon® tube. The suspension was vortexed for 1 minute to separate the conidia, and then centrifuged for 5 minutes, 3000 rpm. The solution was decanted, and the spore pellet resuspended in PZ by vortexing. The spores were pelleted as before, and the wash step repeated. Finally, the conidia were resuspended

in 5 ml PZ and stored at 4 °C. Spore concentration was determined by counting a 10 fold dilution in an haemocytometer. For silica stocks, the spores were pelleted once more and resuspended in 0.5 ml of a sterile solution of 5% skimmed milk (Marvel, Premier Beverages, UK), 4% Na-I-glutamate (freshly made up from separate stock solutions). Three vials containing approximately 0.5g of sterile silica grains (1-3mm, Fluka, Switzerland) were each aliquoted 0.2 ml of the spore suspension, mixed thoroughly by vortexing and stored at 4 °C. Spore suspensions in PZ could be kept for up to six months, and silica stocks were replaced every two years.

2.2.1.2.3 Types of culture

2.2.1.2.3.1 Spore plates

Thick plates of RMG agar (section 2.2.1.1.3) were poured (at least 25 ml of media per 9 cm plate), and allowed to solidify. Plates were either used immediately, or stored at 4 °C and used within 2 weeks. Inoculation was carried out using a meniscus of spore suspension on a sterile wire loop, and spread over the whole plate (this increases the speed at which the culture grows over the whole plate, and therefore the rate of spore production). The spore suspension used was either from a stored spore suspension, or from spores rehydrated by vortexing a small amount of a silica stock in a little PZ. Plates were grown at 30 °C for 7 days (8 days for *A. awamori*) and then harvested (section 2.2.1.2.2).

2.2.1.2.3.2 Shake flasks

Shaking liquid cultures were used to grow large amounts of mycelia without sporulation for transformation, DNA extraction or protein extraction. Depending upon the quantity of mycelia required, either 50 ml of RMG in a 250 ml conical flask or 250 ml RMG in a 2 l conical flask was inoculated with a final spore concentration of 1 x 10^6 spores per ml. Cultures were grown for 18 hours at 30 °C, 300 rpm in a shaking incubator.

2.2.1.2.3.3 Still liquid culture

A method for growing *Aspergillus* cultures for *in vivo* luminometry was developed using still liquid culture in 96 well plates (EG & G Berthold, Germany). Twelve ml of sterile media (either MM with glucose or Vogel's with sucrose (1%), see section 2.2.2.2) was inoculated with 1 x 10^5 spores per ml. Coelenterazine (see section 2.16.2) was added in methanol (MeOH) to a final concentration of 2.5 μM. The final MeOH concentration was not more than 0.1%, which is known not to affect spore germination or hyphal growth (Collis, 1996). Using a 12-channel pipette (Anachem, UK), 100 μl of the inoculated media was added to each well, and the plate covered with a microplate lid (Labsystems, Finland). Cultures were incubated in a humidity chamber in the presence of free water at 30 °C for 24 hours, unless otherwise stated.

2.2.2 *Neurospora crassa*

2.2.2.1 Strain

The wild type strain St Lawrence (St) 74 mating type *A* (74*A*) (Fungal Genetics Stock Centre strain 262) was used for transformation with the apoaequorin gene.

2.2.2.2 Culture media

Neurospora crassa was grown on 1x Vogel's medium N (Vogel, 1956) using either sucrose, glucose, glucose and fructose, or fructose as the carbon source (Table 2.8). For selection of transformants carrying the basta resistance gene (*bar*), Vogel's-N was used with the addition of 200 μg/ml phosphoinothricin to the media. For solid media 2% Oxoid Number 3 agar (Oxoid, UK) was added.

Table 2.8: Vogel's medium N x50 and Vogel's-N x50 stock solutions

Compound	Vogel's N x50	Vogel's -N x50
Na_3 citrate.$2H_2O$	126.7 g	126.7 g
KH_2PO_4	250 g	250 g
NH_4NO_3	100 g	-
$MgSO_4.7H_2O$	10 g	10 g
$CaCl_2.2H_2O$	5 g	5 g
D-Biotin solution[a]	5 ml	5 ml
Trace element solution (see Table 2.9)	5 ml	5 ml
dH_2O	to 1 l	to 1 l

Chloroform (3 ml) was added as a preservative and the solution stored at room temperature. [a] 5 mg of D-biotin was dissolved in 100 ml of 50% volume : volume (v/v) ethanol (EtOH). The solution was stored at 4 °C.

Table 2.9: Trace elements solution for *Neurospora* media

citric acid.$1H_2O$	5.0 g
$ZnSO_4.7H_2O$	5.0 g
$Fe(NH_4)_2(SO_4)_2.6H_2O$	1.0 g
$CuSO_4.5H_2O$	0.25 g
$MnSO_4.1H_2O$	0.05 g
H_3BO_3	0.05 g
$Na_2MoO_4.2H_2O$	0.05 g
dH_2O	to 100 ml

Chloroform (1 ml) was added as a preservative and the solution was stored at room temperature.

2.2.2.3 Culturing *Neurospora crassa*

2.2.2.3.1 Inoculation procedure

Unless otherwise specified, cultures were inoculated using a sterile platinum wire loop. A meniscus of sterile dH_2O in the loop allowed conidia to be taken into suspension during the transfer thereby minimising the risk of conidial dispersal.

2.2.2.3.2 Types of culture

2.2.2.3.2.1 Slants

1 x Vogel's medium N (Table 2.8) with the addition of 2% sucrose (VS medium) was used to maintain stock cultures. Ten ml of unsolidified VS agar was poured into a sterile test tube (150 mm x 15 mm diameter) and allowed to set at an angle of approximately 30°. Inoculated cultures were incubated at 27 °C in continuous illumination for 7 days. Mature cultures could be stored at 4 °C for up to 3 months.

2.2.2.3.2.2 Swirl flasks

To obtain high concentrations of conidia (>1 x 10^9), inoculation was made onto 50 ml of VS agar in a 250 ml conical flask. The medium had previously been solidified by rotating at an angle under a stream of cold water. This resulted in the adhesion of a thin layer of agar to the sides of the flask with a thick layer over the base. Inoculated cultures were incubated at 27 °C in continuous illumination for 6-7 days. Harvested conidia from swirl flasks were used to make long term stock cultures on silica gel as described for *Aspergillus* (section 2.2.1.2.2).

2.2.2.3.2.3 Shaking liquid culture

For bulk conidial germination as required for transformation experiments, conidia were harvested from a swirl flask in 50 ml dH_2O. Conidia were concentrated, washed and finally resuspended in 5 ml dH_2O. The suspension was then added to 150 ml 1x Vogel's medium N (Table 2.8), 1.5% sucrose, in a 500 ml conical flask. This was incubated overnight at 15-18 °C with shaking at 200 rpm.

2.2.2.3.2.4 Still liquid culture

For DNA, RNA, and protein extraction's from mycelium, a conidial inoculation (quantified when necessary) was made into 50 ml VS medium contained in a 250 ml

conical flask. Cultures were incubated standing in darkness at 25 °C for the appropriate time.

2.2.3 Escherichia coli

Table 2.10 shows the *E. coli* strains used in this study:

Table 2.10: Strains of *E. coli* used, their genotypes and references

Strain	Genotype	Reference/ Source
DH5α	supE44, ΔlacU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96, thi-1, relA1	(Hanahan, 1983)
XL1-blue	recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac $^-$, F' [proAB, lacI^q, lacZ□M15, Tn10 (Tet^r)]	Stratagene, UK
BL21(DE3)	F$^-$, omp$^+$, r_B^-, m_B^-, (DE3 is a λ lysogen bearing lacI and lacUV5, gene1)	(Studier *et al.*, 1990)

DH5α and XL-1 blue can be used for the colour-based selection of plasmid insertions. Both strains carry a mutant *lac*Z which codes for the carboxy terminal end of β-galactosidase. The plasmids pTAg and pGEM3z possess the regulatory and first 146 amino acid sequence of this gene adjacent to the multiple cloning site. Following transformation with one of these plasmids the two inactive fragments can combine to produce enzymatically active β-galactosidase. In the presence of IPTG as inducer and X-Gal these colonies appear blue. Those colonies carrying the plasmid with an insertion at the multiple cloning site are incapable of producing potentially active protein and are white.

2.2.3.1 *Escherichia coli* culture media

2.2.3.1.1 Luria-Bertani (LB) medium and agar

LB medium consisted of Difco bacto-tryptone (10 g), Difco yeast extract (5 g) and NaCl (10 g), dissolved in 1 litre of dH_2O. The pH was adjusted to pH 7.2 with 5 M NaOH and the solution was sterilized by autoclaving. LB agar was formed by adding 15 gl^{-1} Difco bacto agar.

2.2.3.1.2 SOB medium

SOB medium was comprised of Difco bacto-tryptone (20 g), Difco yeast extract (5 g), and NaCl (0.5 g) dissolved in 1 litre of dH_2O.

2.2.3.1.3 SOC medium

To 100 ml SOB media, 1 ml 2 M glucose, 1 ml 1M $MgCl_2$, and 1 ml 1 M $MgSO_4$ was added and the solution filter sterilized.

2.2.3.2 Antibiotics

For the selection of XL-1 blue, medium was supplemented with 10 μg/ml tetracycline. For the selection of plasmids carrying an ampicillin resistance gene, 100 μg/ml ampicillin was added to the medium.

2.2.3.3 Colour selection

For colour-selection of pTAg and PGEM3z based plasmids agar was supplemented with 238 mg/ml IPTG and 20 mg/ml X-Gal.

2.2.3.4 *Escherichia coli* cell culture

2.2.3.4.1 Solid medium

LB agar was sterilized and cooled to approximately 50 °C before any supplements were added. The agar was dispensed into sterile 9 cm Petri dishes and allowed to set. To remove condensation from the agar surface and dish lids, plates were

inverted with the lid removed in a drying oven for a brief period. Plates were stored at 4 °C and used within 2 weeks.

Bacterial suspensions were applied to the agar surface using a sterile glass spreader and incubated overnight at 37 °C by which time bacterial colonies (each the progeny of a single cell) were visible.

2.2.3.4.2 Liquid medium

Liquid cultures of *E. coli* were prepared by inoculating a 5 ml volume of LB broth (plus a suitable antibiotic where appropriate) with a single bacterial colony. Cultures were grown overnight at 37 °C with shaking (300 rpm), unless otherwise stated. Larger cultures were prepared by diluting an overnight culture 100-fold in conical flasks containing fresh medium, with an overall capacity 5-10 fold greater than that of the final culture volume to allow for aeration of the media. The growth conditions were as stated in section 2.2.3.4.1.

2.2.3.4.3 Storage of bacterial cultures

For long term storage of bacterial cultures, 4 ml of an overnight culture was mixed with 1 ml of sterile 80% glycerol and aliquots of approx. 1 ml stored in sterile vials at -80 °C. A culture was recovered by streaking onto an agar plate, containing antibiotic if necessary. After overnight incubation at the correct temperature, a single colony was picked and a fresh culture propagated. For short term storage (4-6 weeks) bacteria were stored as streaks on agar plates at 4 °C.

2.3 Extraction and purification of plasmid DNA

2.3.1 Small scale plasmid DNA preparation

Various methods were used, due to different sections of the research being carried out in different laboratories.

2.3.1.1 STET-prep method

This method was adapted from the protocol of Holmes and Quigley (1981). Minipreparations (mini-preps) of plasmid DNA were made from a 1.5 ml aliquot of a 5 ml liquid culture. Cells were harvested in a microcentrifuge (MSE, UK) at 13,500 rpm for 5 min, the supernatant was removed and the cell pellet resuspended in 250 µl STET buffer (8% sucrose, 5% Triton, 50 mM EDTA, 50 mM Tris.Cl, pH 8.0). 10 µl of 10 mg/ml lysozyme was then added to the suspension and vortexed. The tube was placed in a 100 °C dry heating block (Tecam®, UK) for 40 sec, immediately transferred on to ice and 270 µl of 5 M lithium chloride was added. After 30 min incubation the tube was centrifuged at 20,000 g in a microcentrifuge for 5 min at 4 °C, following which the pellet was removed using a sterile toothpick. Ethanol (EtOH) precipitation of the supernatant (section 2.3.3) for 30 min at -80 °C was used to concentrate the DNA, following which it was dissolved in 50 µl TE, pH 8.0 (10 mM Tris.Cl, 10 mM EDTA pH8.0) or dH_2O. Mini-prep DNA was stored at -20 °C.

2.3.1.2 Qiagen® mini-preps

Qiagen mini-preps (Qiagen, UK) use alkaline lysis of the bacterial cells, followed by binding of the plasmid DNA to Qiagen Resin, which is a macroporous anion-exchange resin. Contaminants such as protein, single-stranded DNA and RNA are removed by medium concentration salt washes, and the plasmid DNA eluted in a higher salt buffer (1.25 M NaCl). Finally, the purified plasmid DNA is concentrated and desalted in an isopropanol precipitation. The kit was used following the manufacturer's instructions.

2.3.1.3 Wizard® mini-preps

Wizard® mini-preps (Promega, UK) rely on alkaline lysis of cells and binding of plasmid DNA to a silica membrane in a column. The DNA is washed in a similar manner as Qiagen mini-preps and eluted by centrifuging dH_2O through the column. Plasmid DNA prepared from Wizard mini-preps contains more salt than that found in DNA prepared using the STET-prep method or Qiagen kits. Therefore if a salt-sensitive downstream application was to be used, an EtOH precipitation was carried out to desalt the DNA (section 2.3.3). Otherwise, mini-preps were performed following the manufacturer's instructions.

2.3.2 Large scale plasmid DNA preparation

2.3.2.1 Caesium chloride-ethidium bromide gradient

Maxipreparations (maxi-preps) of closed circular plasmid DNA were obtained by equilibrium centrifugation of cell lysates in caesium chloride (CsCl)/ethidium bromide (EtBr) gradients. The topological constraints of closed circular plasmid limits EtBr binding and its differential density allows it to be resolved as a separate band following ultracentrifugation. Maxi-preps were made from maxicultures of *E. coli* (OD_{600} = 1). Cells were harvested by centrifugation at 6000 rpm, for 10 min, at 4 °C in a Sorvall® GSA rotor using a RCB5 centrifuge (DuPont, USA). The supernatant was decanted and the tubes inverted to remove any remaining culture fluid. The cells were resuspended in 10 ml lysis buffer (25 mM Tris.Cl pH 8.0, 10 mM EDTA, 50 mM glucose, 2 mg/ml lysozyme), split into two SS34 centrifuge tubes (Sorvall®) and left on ice for 10 min. Subsequently, 20 ml total vol. (i.e. 10 ml per tube) of alkaline SDS solution (0.2 M NaOH, 1% SDS) was added, the tubes mixed gently, and left on ice for a further 10 min. To each tube of cell lysate, 7.5 ml 3 M sodium acetate (NaAc), pH 5.5 was added, the tubes mixed by inversion and left on ice for 10 min. The tubes were balanced and centrifuged for 10 min, 15,000 rpm, 4 °C in a Sorvall® SS34 rotor (DuPont, USA). The clear supernatant was decanted into fresh SS34 tubes, an equal volume of isopropanol (approx. 18 ml) added, and

the tubes left for 1 hour at 4 °C, to allow the DNA to precipitate. The tubes were centrifuged for 20 min, 15,000 rpm, 4 °C in an SS34 rotor as before. The supernatant was gently removed and the DNA pellet left to dry for approx. 10 min. The pellets were resuspended in 4 ml TE, then 4 g CsCl and 200 µl 10 mg/ml EtBr added. The solution was transferred into Beckman quick seal ultracentrifuge tubes (Beckman, USA) using a Pasteur pipette, balanced with heavy mineral oil (Sigma) and sealed. The tubes were centrifuged overnight at 45,000 rpm, 15 °C in a T-1270 rotor, in an OTD 50B centrifuge (DuPont, USA). The band of closed circular plasmid DNA was extracted from the gradient using a hypodermic needle (with an extra needle in the top of the tube to allow air in), avoiding the genomic DNA band just above it. Ultra-violet (UV) illumination was used if the band was difficult to see, and to check the position of the genomic DNA band. The EtBr was removed by extraction with TE-saturated butan-1-ol (approx. equal volume added), inverting the tube to mix for two or three min and then replacing the butan-1-ol until it was colourless. To remove CsCl, the DNA was dialysed for 1 hour against TE, the buffer changed and then dialysed overnight. The next day, dialysis was continued, replacing the buffer twice. Final DNA concentration and purity was analysed using a UV spectrophotometer. DNA was stored at -20 °C.

2.3.2.2 Qiagen® maxi preps

Plasmid DNA was purified from maxicultures using a scaled-up version of the Qiagen mini-prep kit (section 2.3.1.2). The technique involved is the same, and the maxi-preps were carried out following the manufacturer's instructions.

2.3.3 Concentrating DNA by EtOH precipitation

Nucleic acids can be concentrated by precipitation with EtOH in the presence of moderate concentrations of monovalent cations. To the DNA solution, 0.1 volumes of 3 M sodium acetate (NaAc) pH 5.5, was added followed by 2 volumes of cold 100% EtOH. Precipitation was promoted by incubating samples at -80 °C for 30

min. The DNA was concentrated by centrifugation at 13,000 rpm for 15 min and the pellet was washed in 70% v/v EtOH. Centrifugation was repeated and after removing the supernatant the pellet was dried under a vacuum of -1 bar. The dried pellet was resuspended in the required volume of TE or dH_2O.

2.3.4 Purification of nucleic acids

For the removal of contaminating proteins and the isolation of DNA fragments from agarose or polyacrylamide gels, DNA was purified either by extraction with phenol: chloroform or by using a suitable kit.

2.3.4.1 UV shadowing oligonucleotides in polyacrylamide gels

This method allows separation of DNA molecules which were only 1 base different in size, and was used to purify synthetic oligonucleotides (oligos) which contain improperly synthesised contaminants which are shorter or longer than the correct sized band. The synthesised oligos were approx. 100 base pairs (bp) long, and for fragments of this size a large 12% polyacrylamide gel is suitable. The gel mix used is shown in Table 2.11.

Table 2.11: 12% polyacrylamide gel mix, 100 ml

40% polyacrylamide (38:2)	30 ml
10x TBE	10 ml
urea	46 g
dH_2O	to 100 ml
10% (w/v) APS (ammonium persulphate)	300 µl
TEMED	30 µl

The gel was poured as described in section 2.12.3 and 50-100 µg DNA in 10% glycerol was loaded per well (more than 100 µg disturbs band borders). Samples were pre-heated at 65 °C for 10 min before loading. One lane was loaded with

buffer (section 2.4) to monitor the speed of migration through the gel. The samples were electrophoresed at 240V until the marker was two thirds of the way down the gel. The gel was then removed from both plates and placed on Saranwrap, and the bands visualised under UV by the shadow they produced on a TLC plate. The correct bands (these are generally the bands with the highest concentration) were then cut out using a sterile scalpel. Each cut out band was then placed in 500 µl TE and incubated at 65 °C overnight. The polyacrylamide was removed by filtering through silanized glass wool as described in section 2.3.4.2, then washing the glass wool with a further 100 µl TE and collecting the eluate by spinning for 5 min at 13,500 rpm. The two eluates were combined and the DNA precipitated in a final concentration of 0.2 M NaCl, 10 mM $MgCl_2$ and 2.5 volumes of cold EtOH. Precipitation was carried out at -20 °C for 30 min, then the DNA collected by centrifugation at 13,500 rpm for 20 min. The pellet was washed with 200 µl cold 70% EtOH and centrifuged at 13,500 rpm for 10 min. This wash was repeated, and then the pellet was allowed to dry briefly, before being resuspended in 50-100 µl TE.

2.3.4.2 Purification of DNA from agarose gels using phenol:chloroform

The required DNA fragment was cut out of the agarose gel (section 2.4) using a sterile scalpel and placed in a 750 µl Eppendorf tube half packed with silanized glass wool. This tube had a pin hole in the base and was placed within a larger 1500 µl tube prior to sterilization. The two tubes were spun at 13,000 rpm for 3 min. The agar remained in the smaller tube and a liquid fraction containing the DNA was collected in the larger tube. Water-saturated phenol (Rathburn Chemicals, UK) was equilibrated using 100 mM Tris.Cl, pH 8.0 and a 1:1 mixture of equilibrated phenol:chloroform made up. An equal volume of this mixture was added to the nucleic acid sample, and mixed to form an emulsion. The two phases were resolved by centrifugation at 13,000 rpm for 5 min and the aqueous phase removed to a clean tube. This step was repeated and to the isolated aqueous phase of the second extraction an equal volume of chloroform was added to remove any contaminating

phenol. Following centrifugation the aqueous phase was removed to a clean tube and precipitated with EtOH (section 2.3.3).

2.3.4.3 Gene Clean II® kit

The protocol used with the Gene Clean® II kit (Bio 101 Inc., USA) followed the manufacturer's recommendations. The agarose was solubilized using sodium iodide and heating the sample to 55 °C for 5 min. The DNA was bound to a silica matrix called Glassmilk®, which was then washed three times with 'New Wash' buffer. The DNA was eluted from the Glassmilk®-DNA complex with the addition of TE, after which the Glassmilk® was discarded.

2.3.4.4 Qiaex II® kit

Qiaex II (Qiagen, UK) purifies DNA from agarose by solubilizing the agarose with chaotropic salts and binding the DNA to Qiaex II silica gel particles. The bound DNA is then washed with a high salt wash to remove residual agarose, followed by two EtOH washes to remove salt contaminants. Traces of EtOH are removed by air drying the Qiaex II particles. The DNA is eluted in a low salt concentration and alkaline pH (10 mM Tris.Cl, pH 8.5). The kit was used following the manufacturer's protocol.

2.3.5 Estimating nucleic acid purity and concentration.

To determine the concentration and purity of DNA and RNA preparations, 1-10 µl of the solution was diluted to 600 µl in dH_2O, and placed in a quartz cuvette with a light path of 1 cm. If the DNA was suspended in TE, 1-10 µl TE in 600 µl dH_2O was used as the blank. The absorption of the solution was measured over a UV wavelength scan of 220 nM to 320 nM using a Beckman® DU-64 spectrophotometer (Beckman, USA). The purity of the nucleic acid preparation was estimated by the ratio OD_{260}/OD_{280} (pure DNA=1.8, pure RNA=2.0). The concentration of the sample

can be calculated from the observation that an OD_{260} = 1 represents approx. 50 μg/ ml for double stranded DNA and 40 μg/ ml single stranded DNA or RNA.

2.4 Agarose gel electrophoresis

Agarose is a linear polymer composed of D-galactose and 3,6-anhydro L-galactose units. Agarose forms a gel in which the pore size and therefore the separation properties are determined by its concentration. A range of concentrations were used depending upon the size of the fragments to be analysed, but generally gels of 0.7-1% agarose were used.

Agarose gels were made up as follows. The required amount of agarose (Severn Biotechnology Ltd, UK) was added to the final volume of 1 x TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) and heated in a microwave oven until the solution was clear and transparent. The solution was allowed to cool to approx. 50 °C and the volume corrected for loss through evaporation with dH_2O. EtBr (10 mg/ml stock solution) was then added to a final concentration of 1 μg/ml and the gel was cast into a gel tray with sample combs in position and allowed to set on a level surface. After setting, the sample comb was removed from the gel along with any tape for sealing the gel tray edges. The cast gel in the tray was then placed in a gel tank which had been filled with 1 x TAE to a level approx. 0.5 cm over the gel surface, and the samples loaded. All DNA samples prior to electrophoresis had sample buffer added at one sixth final volume (6 x sample buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15 % Ficoll, in dH_2O). A standard DNA ladder (Gibco BRL, UK) composed of known band sizes was always loaded in one well (1 μg per well) so that the sizes of the sample DNA fragments could be estimated. Gels were run at 6-15 V/cm.

A range of different sized agarose gels were cast depending upon the length of run required and how many samples were to be loaded. For isolation of DNA fragments from agarose gels (section 2.3.4), low melting agarose (Sigma) was used, as this is easier to solubilize.

2.4.1 Analysis of agarose gels

Following electrophoresis DNA bands were visualised by placing the gel on a UV transilluminater (Hybaid, UK) and the image recorded using either a high performance CCD camera (Cohu, Japan) linked to a video copy processor (Mitsubishi, UK) or a Polaroid camera (Polaroid, UK). DNA fragments to be used in subcloning were excised from the gel using a sterile scalpel, placed into Eppendorf tubes and purified using one of the methods described in section 2.3.4.

2.5 Manipulating DNA using restriction endonucleases and modifying enzymes.

2.5.1 Restriction endonuclease digests

Restriction endonucleases (restriction enzymes) cleave double-stranded DNA at specific sites within or adjacent to a particular sequence. They have a central role in sub-cloning, first in the generation of DNA fragments for incorporation in ligation reactions and second as a diagnostic tool for analysing the products of these ligations.

Typically reaction digests included up to 1 µg of DNA and 0.2-0.3 units per µl (U/µl) of the appropriate restriction enzyme (1 unit is defined as the amount of enzyme required to digest 1 µg of substrate DNA completely in 60 min under the specified conditions for that enzyme). Restriction enzyme buffer (supplied with the enzyme) was present at 1x strength and dH_2O added for a final volume of 20 µl.

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Digest reactions were vortexed then centrifuged briefly and incubated for 1-2 hours at the required temperature (25, 37 or 55 °C). When a double digest using two enzymes was performed, the buffers were checked for compatibility. If both enzymes required different buffers, then the enzyme with the lowest salt buffer was used first, then the buffer optimised for the second enzyme. Alternatively, if the buffers required were quite similar, a compromise buffer was used and the digests carried out at the same time. The reaction products were analysed by gel electrophoresis (section 2.4).

2.5.2 Filling recessed 5' and 3' termini

Recessed 5' and 3' termini created by digestion with certain restriction enzymes, or 3' protruding overhangs generated by the PCR can be filled in or flushed using the Klenow fragment of *E. coli* DNA polymerase I or bacteriophage T4 DNA polymerase (T4 DNA pol), supplemented with the appropriate deoxynucleotide triphosphates (dNTPs). For 3' to 5' exonuclease activity T4 DNA pol is preferred because of its greater 3' to 5' activity.

2.5.2.1 Klenow fragment of DNA polymerase I

After completion of the restriction enzyme (RE) digest, d.NTPs were added to the reaction at a final concentration of 1 mM. One µl of Klenow fragment (5 U/ µl) was added, the tube contents mixed and incubated at room temperature for 15 min. Following incubation, the Klenow and restriction enzymes were inactivated by heating at 75 °C for 10 min and the DNA was then extracted with phenol:chloroform (section 2.3.4.2).

2.5.2.2 Bacteriophage T4 DNA polymerase

The PCR product was mixed with dNTPs to a final concentration of 100 µM and 1.5 µl of T4 DNA pol (1 U/µl) and mixed well. The reaction was incubated at 12 °C for

15 min and the modified DNA recovered by phenol:chloroform extraction (section 2.3.4.2).

2.5.3 Dephosphorylation of vector DNA

Calf intestinal alkaline phosphatase (CIAP) catalyses the hydrolysis of phosphate monoesters from a variety of substrates. CIAP was used to remove the 5' phosphates from linearised plasmids prior to their incorporation in ligation reactions to prevent self-ligation. Up to 1 μg of linearised DNA was treated with 1 μl (1 U/μl) CIAP, 2 μl of x10 CIAP buffer (final concentration: 50 mM Tris.Cl (pH 9.3), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1mM spermidine; supplied with CIAP) in a final volume of 20 μl with dH_2O. The tube was incubated at 37 °C for 30 min and following this CIAP was inactivated by heating at 75 °C for 10 min or removed by phenol:chloroform extraction (section 2.3.4.2).

2.6 Ligation reactions

The joining of linearised DNA (i.e. plasmid vector and gene insert) used bacteriophage T4 DNA ligase. The enzyme catalyses the joining of cohesive or blunted termini of double-stranded DNA molecules by ligating a 5' terminus carrying a phosphate group to a 3' terminus hydroxyl group. To promote the formation of useful recombinant molecules the concentration of the two DNA fragments in the ligation reaction was adjusted to give an insert:vector molar ends of DNA molecules ratio of 3:1 for cohesive end ligation and 10:1 for blunt end ligations. Ligation reactions were also set up with other ratios such as 5:1 and 7:1 for cohesive ends to optimise ligation. 7:1 and 20:1 ratios were set up for blunt end ligations. Control reactions were set up to determine the frequency of plasmid self-ligation, so that it was possible to determine whether the ligation of the insert into the vector had been successful.

The final volume of each reaction was typically 20 µl; this included no more than 300 ng total DNA, 1.5 µl of 1 U/µl T4 DNA ligase and 1x strength ligase buffer (30 mM Tris.Cl (pH7.8), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP); the volume was made up with dH_2O. Ligation reactions were incubated at 15 °C overnight. A 2-10 µl aliquot of each reaction was then transformed into *E. coli* (section 2.9).

2.7 Polymerase Chain Reaction (PCR)

The PCR was used to construct the synthetic apoaequorin gene from oligonucleotides. The method used to amplify the gene fragments from overlapping oligos is known as cross-over PCR.

For PCR of the hybridized product of two oligos, PCR was carried out in a volume of 50 µl with 25 pmoles of each oligo, 1.25 mM of each dNTP, 1x polymerase buffer, and dH_2O to a final volume of 48 µl. Two drops of mineral oil (Sigma) were added to each reaction, and the tubes placed in boiling water for 5 min. Then 2 µl (2U) Taq DNA polymerase was added to each tube and the reactions carried out in a programmed PCR machine (Hybaid Omnigene Thermal Cycler; Hybaid). Controls were set up substituting dH_2O for the oligonucleotides. All pipetting was carried out in a laminar flow hood using sterile filter tips. Melting temperatures (T_m or T_p) for determining the annealing temperatures to be used in PCR were calculated using the equations given below:

Equation 2.1: Determining melting temperature (T_m) for annealing of 20 bases or less

$$T_m = 4(G+C) + 2(A+T)$$

Equation 2.2: Determining melting temperature (T_p) for annealing of 21 to 40 bases

$$T_p = 22 + 1.46(ln), \text{ where } ln = 2(G+C) + (A+T)$$

For both equations, G, C, A and T refer to the number of these bases present in the sequence.

For the PCR of a single DNA molecule from two hybridized products obtained from two PCR reactions as carried out above, the method used was essentially the same, except that the reaction volume was 100 µl, primers were added (100 pmoles of each) and the assumed products of the first PCR reactions added. An equal ratio of each PCR reaction was added, based upon the initial amount of each oligo present in the reactions.

Full details of reaction conditions and gene synthesis using PCR are given in Chapter 3. Following thermal cycling the reaction products were examined by gel electrophoresis (section 2.4).

2.8 DNA Sequencing

Sequencing of DNA uses replication of single-stranded DNA using a DNA polymerase, incorporating labelled nucleotides during the reaction so that the DNA can be visualised (using autoradiography for ^{35}S-labelled dNTP or monitoring fluorescence for fluorescent-tagged dNTPs). To know the sequence of the DNA, di-deoxy nucleotides (ddNTP) are added to the reaction. Once a di-deoxy nucleotide is incorporated into the DNA, the polymerase can no longer add any further nucleotides, and therefore the reaction is stopped. If only one type of di-deoxy base is added to a reaction (e.g. ddATP), the replication of the DNA will be stopped every time that a ddATP is incorporated. By carrying the reaction out four times, each one in the presence of a different di-deoxy nucleotide, every possibility will be covered.

If the reactions are then run on a 6% polyacrylamide gel (section 2.12.3), each differently terminated DNA molecule will be a different size, and therefore will be separated out in the order that the ddNTP's were incorporated. Visualisation of the gel (using a suitable method) will then give the sequence of the DNA by comparing each ddNTP reaction.

2.8.1 Manual sequencing using ^{35}S-labelled nucleotides

The T7 Sequenase version 2.0 DNA sequencing kit (Amersham Life Science, UK) was used for manual sequencing. For each reaction carried out, 3 µg of template DNA was used. The volume of the template DNA was adjusted to 32 µl with dH_2O, and 8 µl of denaturing solution (1 g NaOH pellets, 50 µl 0.5 M EDTA, pH 8.0 in 20 ml dH_2O, made up fresh each time) added, and the DNA denatured at 37 °C for 15 min. Then 4 µl 2 M ammonium acetate, pH 4.5, and 2 volumes EtOH were added, and the tube left on dry ice (-70 °C) for 15-30 min to precipitate the DNA. The precipitated, denatured DNA was spun down at 15,000 rpm at 4 °C for 20 min, washed with 70% EtOH and centrifuged once more. The pellet was then air-dried and resuspended in 14 µl dH_2O. The resuspended DNA was then transferred to a sterile 96 well siliconised plate (Falcon, Becton Dickinson, New Jersey, USA). Sequencing primer was then annealed to the single-stranded template DNA by the addition of 2 µl 5x reaction buffer, 1 µl primer (10 pmoles) and incubating at 37 °C for 10 min. Sequencing reactions were then carried out as follows. 2.5 µl of each stop reaction (ddATP, ddCTP, ddGTP, ddTTP) was added to individual wells in the same column as the annealing reaction. 5.5 µl of reaction mix (prepared during annealing reaction, containing: 0.5 µl [α^{35}S] dATP (10 µCi/µl, 10 µM dATP), 1 µl dithiothreitol (DTT), 0.4 µl dNTPs, 1.75 µl enzyme dilution buffer, 1.6 µl dH_2O and 0.25 µl sequencing enzyme per annealing reaction) was added to the annealing reaction, and 3.5 µl of the sequencing reaction was then added to each of the stop wells containing the ddNTPs. Reactions were left at 37 °C for 10 min, then 4 µl of stop reaction buffer was added to each of the four wells. Sequencing reactions were

either immediately run on a pre-poured, pre-warmed 6% polyacrylamide gel (section 2.12.3), or stored at -20 °C.

To overcome compression artifacts in the sequencing gel results which were due to the very GC rich nature of the synthetic apoaequorin gene, alternative dNTP and ddNTP mixes were used. These used a guanidine analogue called inosine (base I), which can only form two of the normal three hydrogen bonds between guanidine and cytosine, therefore weakening the structure, allowing the sequencing oligos to run correctly on the gel.

2.8.1.1 Assembly and running of polyacrylamide sequencing gels

A Biorad Sequi-Gen II Sequencing Cell apparatus (38 x 50 cm) was used for running sequencing gels. Prior to use, the plates, combs and spacers were washed with a detergent, rinsed with dH_2O and then washed with 95% EtOH. Plates were then dried using dust-free tissues. Occasionally (approx. after every 20 runs), the back plate was silanized using Repelcote (Sigma), allowed to dry and washed thoroughly as described above. The dry apparatus was assembled for pouring the gel as per the manufacturer's instructions. A 6% polyacrylamide gel was made up using the mix described in Table 2.12 by adding 680 µl 10% APS (ammonium persulphate) and 53.3 µl TEMED to 100 µl of gel mix to allow polymerisation. The gel was poured using a 50 ml pipette, holding the gel apparatus at an angle, avoiding introduction of air bubbles. The combs were inserted upside down and the gel left to polymerise overnight with the top of the gel wrapped in Saranwrap.

Table 2.12: 6% polyacrylamide gel mix: 500 ml

40% polyacrylamide (38:2)	75 ml
10x TBE	50 ml
urea	230 g

The solution was filter-sterilised, de-gassed, and stored at 4 oC for up to two weeks.

The gel kit was then assembled for running as per the manufacturer's protocol, using 1x TBE buffer for the upper and lower reservoirs. The gel was pre-warmed at 1500 V (60 W) for 30-60 min. Samples were heated at 95 °C for 5 min prior to loading. 3.5 µl of each reaction was loaded and the gel electrophoresed at 1500 V (60 W) until the first blue band reached the bottom of the gel (~2.5 hours). Then, in empty wells, a second aliquot of each reaction was loaded and the gel ran until the second blue band of the first loading reached the bottom of the gel (~5.5 hours). This allowed the sequences to be read over a greater distance. The gel was then switched off and the buffer disposed of in a safe manner. The two plates of the gel were carefully prised apart, keeping the gel attached to the front plate. The gel and plate were immersed in fixing solution (10% MeOH, 10% acetic acid) for 30 min. The gel was then carefully drained, and a piece of 3MM blotting paper slightly larger than the size of the gel was then laid over the gel avoiding air bubbles between the paper and the gel. Using the blotting paper, the gel was pulled away from the plate, turned over and covered in Saranwrap on the exposed side. The gel was then placed in a Biorad Model 583 gel dryer, blotting paper side down, and dried at 80 °C (~1.5 hours). The Saranwrap was removed from the dried gel, and the gel placed in an autoradiography cassette, blotting paper side down. An autoradiography film was placed over the top of the gel in the dark room, and the film exposed overnight. Exposed film was developed using an automatic film developer (Kodak, UK).

2.8.2 Automated sequencing

Automated sequencing was used to verify manual sequencing results of putatively correct synthetic apoaequorin gene clones. This was done at Zeneca Pharmaceuticals, Macclesfield, UK, using an ABI Prism sequencer with fluorescent dye-labelled di-deoxy nucleotides and a thermo sequenase DNA polymerase. The only work that was necessary was to prepare a template DNA and primer mix. Automated sequencing is very susceptible to contaminating salt, and therefore

Qiagen maxi-prep (section 2.3.2.2) DNA was used. After preparing the DNA, it was given a further EtOH precipitation (section 2.3.3) with two 70% EtOH washes. For each sequencing reaction a mix of 0.5 µg template DNA and 3.2 pmoles of primer in a total of 12 µl dH_2O was prepared.

2.9 *Escherichia coli* transformation

2.9.1 Preparation of $CaCl_2$/ $RbCl_2$ competent cells

A single colony was picked from a freshly grown plate and used to inoculate 5 ml of pre-warmed LB. The culture was grown at 37 °C for approx. 2 hours. This was then sub-cultured 1:20 into 100 ml LB and grown until the OD_{550} reached 0.48 (approx. 1.5 hours). The culture was immediately chilled on ice for 5 min and the cells harvested by centrifugation at 3000 rpm, 4 °C for 10 min, in 50 ml Falcon tubes in a benchtop centrifuge. The supernatant was discarded and the cells resuspended in two fifths of the initial volume using TfbI (30 mM potassium acetate, 0.1 M $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% v:v glycerol, pH adjusted to 5.8 with 0.2 M acetic acid). Cells were left on ice for 5 min, then pelleted as before. The supernatant was discarded and the cells resuspended in one tenth of the volume of TfbI using TfbII (10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% v:v glycerol, pH adjusted to 6.5 with 1 M KOH) and the cells left on ice for 15 min. 220 µl aliquots of cells were then pipetted into 1.8 ml cryotubes and stored at -70 °C. Prior to their use, cells were defrosted slowly on ice.

2.9.2 Transformation of $CaCl_2$/ $RbCl_2$ competent cells

For transformation, 2-10 µl of DNA was aliquoted into a 12 ml round bottomed polypropylene Falcon tube which had been left on ice. 40 µl of thawed competent cells were added to the DNA, and mixed gently by tapping the tube. The cells were then incubated on ice for 15-30 min. The cells were heat-shocked in a 42 °C water

bath (Grant, UK) for exactly 45 sec and then immediately put on ice for 2 min. 450 µl of LB + 20 mM glucose was added to the tube prior to a 45 min outgrowth period at 37 °C, 200 rpm. The cells were then plated onto LB agar plates containing the appropriate antibiotics using approx. 220 µl of cells per plate. Plates were incubated overnight at 37 °C.

2.9.3 Preparation of electro-competent cells

Electrotransformation has been estimated to be 10-20 times more efficient than transformation obtained with maximally competent cells prepared by chemical methods (Dower *et al.*, 1988).

Overnight 5 ml cultures of DH5α were used to inoculate individual flasks of 250 ml LB (one overnight culture per flask), which were incubated at 37 °C, 300 rpm until the OD_{600} was 0.6-0.9 (approx. 4 hours). The cell cultures were chilled on ice for 30 min and the cells were harvested by centrifugation at 4 °C at 3600 rpm. The media was removed, the cell pellets resuspended in 250 ml ice cold dH_2O and centrifuged again. The cells were then resuspended in 125 ml ice cold dH_2O, the cells from two flasks pooled and centrifuged. This was repeated, resuspending the cells in 100 ml ice cold dH_2O and pooling two centrifuge pots. Cell pellets were then resuspended in 50 ml ice cold dH_2O, transferred to 50 ml Falcon tubes and centrifuged in a benchtop centrifuge at 4 °C for 10 min, 3000 rpm. The cell pellets were resuspended in 20 ml 10% glycerol, centrifuged as before, and finally resuspended in 5 ml 10% glycerol. The cell suspension was divided into 100 µl aliquots in Eppendorf tubes and snap frozen in liquid nitrogen and stored at -70 °C until use.

2.9.4 Transformation by electroporation

Stored electro-competent cells were defrosted slowly on ice and electroporation cuvettes (0.1 cm width) were also chilled on ice. A 1-5 µl aliquot of a DNA solution

(preferably in dH_2O) was added to the 50 µl of cell suspension, mixed gently and then transferred to a cuvette using a Pasteur pipette. The cuvette was placed in the safety chamber of the pulse controller (Bio-Rad, UK) ensuring contact with the electrodes, and a single 2.5 kV square electrical pulse was administered from the adjoining gene pulser (Bio-Rad, UK). The characteristics (resistance 200 Ω, capacitance 25 µF, time constant 4-5 msec, field strength 25 kV/cm) of this pulse had previously been shown to be optimal for *E. coli*. The cuvette was immediately removed from the apparatus and 1 ml of SOC medium was added. The cells were gently resuspended in this using a Pasteur pipette, transferred to a 15 ml polypropylene tube and incubated at 37 °C for 1 hour shaking at 225 rpm. Individual aliquots of 200 µl of the cell suspension were applied to LB plates supplemented with the appropriate antibiotics and incubated overnight at 37 °C.

2.10 Transformation of *Aspergillus*

This protocol uses protoplasts and $MgSO_4$ for transformation, and is based upon the method described by (Punt and van den Hondel, 1992).

2.10.1 Producing protoplasts

Mycelia was harvested from a swirl flask (section 2.2.1.2.3.2) by filtering the mycelia on a double layer of sterile muslin and washing with 0.6 M $MgSO_4$. Excess fluid was removed by squeezing the mycelia carefully, then it was transferred to a pre-weighed 50 ml Falcon tube and placed on ice. The mycelia was then weighed and resuspended in 5 ml/g mycelia using osmotic medium (OM: 1.2 M $MgSO_4$, 10 mM Na-phosphate, pH 5.8), containing 1 mg/ml Novozyme 234 (Novo Nordisk, Denmark) and 1.2 mg/ml BSA. This was incubated in a 250 ml conical flask at 30 °C for 1 hour shaking at 80 rpm. Protoplasting was followed by removing samples at intervals and examining them under the microscope. A large number of protoplasts were usually visible after approx. 1 hour. Once pieces of broken mycelia started to

appear in the protoplast suspension, the protoplasts were harvested by filtering through a double layer of sterile muslin and collecting the protoplasts on ice. The filtrate was aliquotted into 30 ml fractions in 50 ml Falcon tubes, and 20 ml ice cold STC (1.2 M sorbitol, 10 mM Tris.Cl, pH 7.5, 50 mM $CaCl_2$) added. Protoplasts were pelleted in a benchtop centrifuge (10 min, 4 °C, 3000 g), the pellets combined and resuspended in 50 ml STC. Protoplasts were pelleted once more, and the wash step repeated. Finally the protoplasts were resuspended in a small volume of STC and the concentration determined by counting in a haemocytometer. The suspension volume was then adjusted to give a final concentration of 1 $x10^8$ protoplasts/ml. Dilutions of the protoplast suspension were plated out on osmotically protected (section 2.2.1.1.4) and minimal media (section 2.2.1.1.1) to determine protoplast viability and spore concentration. The plates incubated at 30 °C for 4-5 days.

2.10.2 $MgSO_4$/PEG transformation

Between 5-20 µg of DNA (in a maximum of 10 µl) was placed in the bottom of a sterile 14 ml polypropylene tube and 1 µl 1 M aurintricarbonic acid (ATA, a DNase inhibitor) added. One tube was set up without DNA as a control. 100 µl of protoplasts (1 $x10^7$) were then added, and the tubes incubated on ice for 25 min. PEG solution (60% w/v PEG 4000, 10 mM Tris.Cl, pH 7.5, 50mM $CaCl_2$) was then added, two drops at a time from a wide-mouthed pipette, gently swirling the tube by hand to mix. A total of approx. 1.45 ml was added in this manner (approx. 14 drops), and the tubes gently rolled until the solutions had mixed thoroughly. The PEG/protoplast mix was incubated at room temperature for 20 min. If room temperature was greater than 20 °C, then the tubes were placed in a 20 °C water bath. Each tube was then carefully filled with STC and mixed gently. Protoplasts were pelleted by centrifuging for 10 min, 3000 g at 4 °C in a benchtop centrifuge, and the supernatant decanted. The pellet was resuspended in the remaining solution (approx. 200-300 µl), and the protoplasts plated (two plates per transformation) using osmotically protected selective media (section 2.2.1.1.4). Plates were incubated at

30 °C for 7 days. The volume of the no-DNA control was determined, and a spore count and viable protoplast count done by plating dilutions on both osmotically protected plates (section 2.2.1.1.4) and MM plates without selection (section 2.2.1.1.1). Plates were incubated at 30 °C for 4-5 days.

2.10.3 Transformant purification

Protoplasts were likely to contain more than one nuclei, and therefore transformants were probably heterokaryons. Consequently, the transformants had to be purified by serial isolation of spores.

Transformants were picked after they had started sporulating. Positive transformants were seen to grow strongly on the selection media, and the largest colonies were chosen. Colonies were picked by collecting spores on a moistened sterile toothpick and plated in triplicate onto MM, MM + acrylamide and MM + acetamide plates (sections 2.2.1.1.4 and 2.2.1.1.1). Plates were incubated at 30 °C, and after 4 days colonies which grew healthily on all 3 plates were picked from the MM + acrylamide plate and streaked out on a new MM + acrylamide plate to isolate single colonies. The plates were incubated at 30 °C for 4 days, after which isolated colonies which had grown from a single spore were picked and streaked out again. Again, after 4 days, a single colony was picked and plated out on an RMG plate (section 2.2.1.1.3), and the spores harvested as described in section 2.2.1.2.2.

2.11 Transformation of *N. crassa*

Plasmid DNA was transformed into *N. crassa* by electroporation of protoplasts, using a method developed by (Collis, 1996).

2.11.1 Protoplast formation

Protoplast formation was based on the method of (Vollmer and Yanofsky, 1986) using germinated macroconidia (referred to as conidia). Conidia were isolated from a swirl flask (section 2.2.2.3.2.2) by adding 30 ml of dH_2O to the culture swirling gently and then pouring the conidial suspension through a double layer of sterile muslin into a 50 ml screw cap polypropylene tube. Conidia were washed twice in dH_2O by centrifugation at 2500 rpm for 3 min (MSE Mistral 1000, UK) and finally resuspended in 5 ml dH_2O. The conidia were germinated in a shaking liquid culture (section 2.2.2.3.2.3). The germinated conidia were washed twice in dH_2O and then resuspended in 10 ml of 1 M sorbitol (osmotic protectant) and placed in a sterile 250 ml flask. Five ml of 5 mg/ml filter sterilized Novozyme 234 (Novo Nordisk, Denmark) in 1 M sorbitol was added to the protoplast suspension and this was incubated at 30 °C for 1 hour shaking at 100 rpm. The protoplast mix was decanted into a 50 ml polypropylene tube and the volume made up to 50 ml with 1 M sorbitol and centrifuged at 2500 rpm for 3 min to concentrate the cells. After removing the supernatant the pellet was resuspended in 50 ml of 1 M sorbitol and centrifuged. The pellet was then resuspended in 50 ml 1 M sorbitol.

2.11.2 Transformation by electroporation of protoplasts

The methodology of this procedure was based upon protocols for *N. crassa* germinated conidia (Chakraborty and Kapoor, 1990) and *S. cerevisiae* transformation (available from Biorad, UK), and from studies on protoplast regeneration (Selitrennikoff and Bloomfield, 1984).

Protoplasts were produced as in section 2.11.1. Following an additional wash in cold 1 M sorbitol the cells were resuspended in 1-5 ml of cold 1 M sorbitol. An aliquot of 6×10^6 chilled protoplasts (approx. 50 µl) was mixed with 1 µl (2 µg) of chilled DNA and transferred to a sterile chilled 0.2 cm width electroporation cuvette. The cuvette was placed in the safety chamber of the Biorad Gene Pulser and a square electrical

pulse administered (resistance 200 Ω, capacitance 25 μF, time constant 4-5 msec, field strength 12.5 kV). The electroporated cells were immediately transferred to 1 ml of 1x Vogel's medium N, 1 M sorbitol, 0.5% glucose, 0.5% fructose and incubated at 30 °C shaking at 100 rpm for 4 hours to allow cell wall regeneration. A 300 μl aliquot of the cell suspension was then plated directly on to 1x Vogel's-N, 1 M sorbitol, 0.5% fructose, 0.05% glucose, 2% sorbose, 0.5% proline, 200 μg/ml phosphoinothricin, and incubated at 25 °C for 6 days.

2.11.3 Formation of homokaryons

Macroconidia are mainly multinucleate (average =2-3 nuclei), and therefore it was assumed that most of the primary transformants would be heterokaryons, i.e only one nucleus out of several would be transformed. As *N. crassa* is haploid a transformed genome can be purified by extracting a homokaryon state. This was achieved by serial isolation of phosphoinothricin-resistant macroconidia.

Conidia from a primary transformant were inoculated into 10 ml of dH_2O, the suspension was mixed by vortexing and a 100 μl aliquot of the suspension was spread on to a 1x Vogel's-N, 0.05% glucose, 0.05% fructose, 2% sorbose, 200 μg/ml phosphoinothricin agar plate. Plates were incubated at 25 °C in darkness for 3 days. Isolated colonies from these plates were inoculated onto VSH slants and incubated in continuous illumination at 27 °C for 7 days to obtain sufficient conidia for the second round of single colony isolation. The plating and colony isolation procedure was repeated 3 times for individual primary transformants. After 3 serial isolations the probability of the strain remaining heterokaryotic is likely to be 10% or less.

2.12 *in vitro* protein manipulation and detection

2.12.1 Protein extraction

2.12.1.1 Preparation of whole cell extract from *E. coli*

Whole cell extracts from *E. coli* were made to purify an apoaequorin:His-tag fusion protein which was made using the His-tag expression vector, pET16b, and expressed in BL21 cells.

An overnight culture of BL21 transformed with pET16b containing the apoaequorin:His-tag fusion gene was used to inoculate a large culture by a 1:20 dilution. The cells were grown until the OD_{600} reached 0.6-0.8, and then expression of the fusion gene was induced with the addition of IPTG to a final concentration of 0.5 mM. Cells were grown for a further 3 hours, and then harvested in a Beckman JA-20 (JA-14 rotor) centrifuge at 6000 rpm, 4 °C, for 15 min. Cells were resuspended in a minimum volume of wash buffer (Table 2.17), and then lysed by sonication using a Lucas Dawes Ultrasonics Soniprobe, with probe size 60 mm x 12 mm diameter. Sonication was carried out on output setting 5, and pulsed for 40% of each second over a 4 minute period in an ice cold water bath. Cell debris was then pelleted at 16,000 rpm for 30 minutes at 4 °C in a Beckman JA-20 (JA-21 rotor) centrifuge. The supernatant was retained and stored at -20 °C prior to protein purification (section 2.12.5).

2.12.1.2 Preparation of whole cell extract from filamentous fungi

Whole cell extracts were used to compare the expression levels of apoaequorin obtained in different *N. crassa* transformants. *Aspergillus* transformants were also used as a positive control, and for comparison of expression levels by SDS-PAGE and Western blotting.

Cultures were grown as described in section 2.2.2.3.2.4 for *N. crassa* and in section 2.2.1.2.3.2 for *Aspergillus*. Mycelia were harvested by sieving from the media using

a double layer of sterile muslin. The collected mycelia were washed with PZ (0.8% NaCl), gently squeezed dry and immediately frozen in liquid nitrogen. A mortar was filled with liquid nitrogen (one third full), the mycelia added and ground to a fine powder. The mycelial powder was transferred to a pre-weighed and cooled polypropylene tube and stored in liquid nitrogen until all samples had been ground. The samples were then weighed and suspended in extraction buffer (10 mM EGTA, 50 mM Tris.Cl, pH7.4, 500 mM NaCl, 10 mM β-mercaptoethanol, 0.1% BSA in HPLC H_2O). 1 ml of buffer was added for every 0.2 g of mycelia. Samples were vortexed for a minimum of 30 sec, and placed on ice until all samples were ready. The extraction suspensions were then decanted into Eppendorf tubes and the insoluble material pelleted in a microcentrifuge at 20,000 g for 5 min. The supernatant was retained and stored at -20 °C.

2.12.2 Estimation of protein concentration

The concentration of a protein in a solution was estimated using the modified method of Bradford (1976) by following a Biorad standard assay procedure. BSA standards of 0, 2, 4, 6, 8, 10 and 12 µg were prepared in 800 µl of dH_2O and 200 µl of Biorad protein assay dye-reagent was added. Each sample was mixed by gentle inversion, taking care not to cause foaming, and after a period of 5 to 20 minutes the optical density of the solution was read at 595 nm (OD_{595}) in a spectrophotometer. For each BSA standard solution, triplicate measurements of the OD_{595} were obtained and the average reading was plotted against concentration to give a standard curve. The protein concentration of the solution of interest was then determined by appropriate dilution of the sample and addition of 800 µl of this solution to 200 µl of the dye-reagent. The optical density was then determined as outlined above. This reading was used in conjunction with the standard curve to determine the concentration of protein in the solution of interest.

2.12.3 SDS-polyacrylamide gel electrophoresis of protein samples

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by (Laemmli, 1970). The procedure dissociates proteins into their individual sub-units and subsequently separates them according to their size.

Proteins were resolved on either a 16 cm x 20 cm x 1.5 mm or 7 cm x 8 cm x 0.75 mm SDS-polyacrylamide gel, using a PROTEAN II xi Slab Gel or MINI-PROTEAN II Dual Slab Gel apparatus, respectively (Biorad, UK). A 15% (v/v) resolving gel was routinely used and the stacking gel was used at a 4% (v/v) polyacrylamide concentration. The compositions of the protein gels are shown in Tables 2.13, 2.14 and 2.16 (the quantity of solutions indicated were designed for the formation of two 7 x 8 cm gels; the values in parentheses reflect the quantities of components required to make a 16 x 20 cm gel).

Table 2.13: Stacking Gel (4% [v/v] polyacrylamide)

30% (w/v) acrylamide/ bisacrylamide (29:1)	1.4 ml (3.9 ml)
Tris.Cl pH 6.8, 0.5 M	2.3 ml (7.5 ml)
20% (w/v) SDS	50 µl (150 µl)
dH_2O	6.2 ml (18.3 ml)
10% (w/v) APS (ammonium persulphate)	100 µl (300 µl)
TEMED	10 µl (30 µl)

Table 2.14: Resolving Gel (15% [v/v] polyacrylamide):

30% (w/v) acrylamide/ bisacrylamide (29:1)	5.0 ml (25.0 ml)
Tris.Cl pH 8.8, 1.5 M	2.5 ml (12.5 ml)
20% (w/v) SDS	50 µl (250 µl)
dH_2O	2.4 ml (12.0 ml)
10% (w/v) APS	50 µl (250 µl)
TEMED	5 µl (25 µl)

Table 2.15: Laemmli (Loading) Sample Buffer (10x) for SDS-polyacrylamide gels:

25 mM Tris.Cl, pH 6.8
2% (w/v) SDS
10% (v/v) Glycerol
0.2 M β-mercaptoethanol
0.002% (w/v) Bromophenol Blue

Table 2.16: 5x Running buffer for SDS-polyacrylamide gels:

Glycine	144 g
Tris-base	30 g
SDS	5 g
dH_2O	to 1 litre

The resolving gel was poured between two glass plates, overlaid with dH_2O and allowed to polymerise. Once the resolving gel was set, the layer of water was removed and the stacking gel was poured. A comb was immediately inserted into the stacking gel, ensuring no bubbles were trapped around the teeth of the comb. Once the stacking gel had polymerised, it was used immediately or stored, covered in cling-film to prevent dehydration, at 4 °C for up to 24 hours before use.

Samples to be loaded were mixed with 2x Laemmli (or Loading) sample buffer (LSB) and boiled for five minutes prior to loading. Samples were loaded (15-50 µl, depending upon the gel used) into individual wells and the proteins electrophoresed in 1x running buffer at 25-50 mA for 2 hours (MINI-PROTEAN II) or 12 mA for 12 hours (PROTEAN II). Molecular weight standard markers (Biorad) were also added to allow molecular weight determinations of polypeptides seen on the gel.

2.12.4 Staining SDS-polyacrylamide gels with Coomassie Brilliant Blue

Polypeptides were visualised by staining with Coomassie Brilliant Blue. Once the polypeptides had been separated by electrophoresis, the gels were immersed in a fixing/staining solution (0.1% [w/v] Coomassie Brilliant Blue R250 (Biorad)

dissolved in destaining solution (see below) and filtered prior to use. Gels were left to stain on a shaking platform for 30-60 minutes at room-temperature. Excess stain was then removed from the gel by soaking it in a destaining solution (methanol, glacial acetic acid and dH_2O (45:10:45; v/v) for 4-8 hours at room temperature, until polypeptide bands could be seen clearly. For storage, gels were dried overnight at room temperature between two sheets of cellophane, in a frame used to keep the cellophane taught.

2.12.5 Purification of histidine-tagged AeqD using a nickel-nitrilotriacetic acid (NTA) column

The nickel (Ni^{2+}) groups in Ni^{2+}-NTA resin (Qiagen) are capable of binding the imidazole ring of histidine amino acids. Proteins containing a His-tag (added when expressed using pET-16b) can therefore be selectively bound and separated from non-His-tagged proteins with relative ease, using either a pH gradient or imidazole gradient. For this study an imidazole gradient was used, which competes for the Ni^{2+} groups in the resin and therefore elutes proteins in order of their binding strength to the Ni^{2+}-NTA resin.

Table 2.17: Ni^{2+}-NTA column wash buffer:

50 mM Na-phosphate, pH 8.0
300 mM NaCl
0-180 mM imidazole

Table 2.18: Ni^{2+}-NTA column elution buffer

50 mM Na-phosphate, pH 8.0
300 mM NaCl
240 mM imidazole

All buffers were made up in dH_2O. Various concentrations between 0 mM and 120 mM imidazole were used for the wash buffer (Table 2.17).

All steps were performed at 4 °C. 1.5 ml of a dilute suspension of resin was transferred to a column and allowed to settle (forming a 1 ml column). The column was washed with 10 ml of dH_2O and equilibrated using a further 10 ml of wash buffer (Table 2.17, 0 mM imidazole). 5 ml of cleared cell lysate (section 2.12.1.1) was loaded onto the column. To remove non His-tagged proteins, the column was washed with the following concentrations of imidazole:

a) 20 ml wash buffer (0 mM imidazole)

b) 40 ml wash buffer (20 mM imidazole)

c) 20 ml wash buffer (120 mM imidazole)

d) 10 ml wash buffer (180 mM imidazole)

Each fraction was collected and stored at 4 °C.

The His-tagged protein was eluted by the addition of 10 ml of elution buffer (Table 2.18), and the fraction stored at 4 °C until further use. The purification of the His-tagged protein was monitored by examining the composition of column fractions by SDS-PAGE (section 2.12.2). Imidazole containing samples were heated to 40 °C for 10 min prior to loading, instead of boiling for 5 min. The 180 mM imidazole wash and the elution fraction were TCA-precipitated prior to SDS-PAGE analysis to concentrate the protein samples (section 2.12.6). Gels were run at 25-50 mA for 2 hours, stained with Coomassie Brilliant Blue stain and then destained.

The Ni^{2+}-NTA columns could be recharged after elution of the proteins. This was done by washing the column through with 5 volumes wash buffer (0 mM imidazole), then loading the column with 5 volumes 50 mM $NiSO_4$ and leaving it to stand for 10 min. The column was then washed through with 10 volumes wash buffer (0 mM imidazole). In this way a column could be re-used many times. Columns were stored at 4 °C in 30% (v/v) EtOH.

2.12.6 Concentrating protein samples

2.12.6.1 Concentration of protein samples using trichloroacetic acid (TCA)

TCA was used to concentrate His-tag purified samples for SDS-PAGE. Typically, 10 µl of each column fraction was diluted to 100 µl with dH_2O and an equal volume of 10% TCA (w/v) was added. The sample was left on ice for 20 minutes and then centrifuged at 13,500 rpm in a microcentrifuge for 15 minutes. The pellet was washed in 100 µl ice cold EtOH, which was decanted off. The pellet was desiccated and resuspended in 20 µl 2x LSB.

2.12.6.2 Concentrating protein samples with Vivaspin columns (Vivascience Ltd., UK)

His-tagged purified protein was concentrated for antibody production using 15 ml Vivaspin columns. These contained a polyethersulphone membrane which had a molecular weight cut-off of 10 kDa. The columns were used following the manufacturer's protocol. Typically, a 10 ml column fraction could be concentrated down to less than 500 µl in two centrifuge spins using the second spin to wash the concentrated protein with PBS (Dulbecco's phosphate buffered saline, pH 7.4: 800 mg NaCl, 20 mg KCl, 144 mg Na_2HPO_4, 24 mg KH_2PO_4 in 100 ml). Samples were then dialysed against PBS overnight at 4 °C to remove any traces of imidazole. Protein concentration was estimated as described in section 2.12.2. Yields from Vivaspin columns were approx. 80%.

2.12.7 Immunodetection of polypeptides

Immunological detection of proteins was performed using a Western blotting procedure. Proteins were first transferred (blotted) to a solid support (Towbin *et al.*, 1979) *e.g.* PVDF membrane and the blot was exposed to sera raised against that protein, in mice. Bound sera was then detected using anti-mouse immunoglobulin antisera conjugated to alkaline phosphatase, which allows visualisation of the protein band by chromogenic staining when the enzyme is exposed to its substrate (Knecht and Diamond, 1984).

2.12.7.1 Transfer of proteins to PVDF membrane

The procedure for transferring proteins to PVDF membranes from polyacrylamide gels was essentially that of (Towbin *et al.*, 1979), with modifications suggested by the manufacturers of the apparatus used. A Biorad "Trans-blot cell" was utilised for the transfer of polypeptides from the polyacrylamide gel to a PVDF membrane.

Once SDS-PAGE had been performed, 3 pieces of 3MM Whatman paper were cut to the size of the gel (16 x 20 cm) and were soaked in transfer buffer (10 mM CAPS containing 10% [v/v] methanol, pH 11.0). The gel (pre-soaked in transfer buffer for 15 minutes) was then carefully transferred to the filter paper, ensuring no bubbles were trapped between the gel and the paper, and both were laid onto a transfer buffer soaked pad. An appropriately sized piece of PVDF membrane (Millipore) (previously soaked in methanol for 15 seconds, dH_2O for 2 minutes, and transfer buffer for at least 15 minutes) was carefully placed over the gel, again taking care to avoid bubbles. A second stack of 3 sheets of buffer-soaked filter paper was placed over the membrane and the sandwich was completed by a second soaked filter pad. The entire sandwich was placed into a holder and immersed into a gel tank containing transfer buffer, making sure that the membrane "side" of the sandwich was closest to the anode. Polypeptides were then transferred from the gel to the membrane by electrophoresis at 45 V for 24 hours at 4 °C. After blotting the polypeptides, the membrane was used immediately for immunodetection experiments.

2.12.7.2 Immunodetection of polypeptides

The detection of polypeptides followed the method of (Knecht and Diamond, 1984). A blotted membrane was shaken for 1 hour at room temperature in 100 ml of 5% (w/v) milk powder dissolved in 1x TBS (Tris-buffered saline). The milk proteins blocked sites on the membrane not already filled by proteins from the gel sample. 100 µl of mouse sera (raised against purified His-tagged AeqD protein - see section 2.12.5) was added to the milk solution and shaken for at least 5 hours (usually overnight), at room temperature. After this time, the membrane was washed six

times with 1x TBS, each wash involving shaking at room temperature with 100 ml of buffer for 10 minutes. Following washing, the membrane was shaken, at room temperature, in 15 ml 1x TBS containing a 1:1,000 dilution of alkaline phosphate-conjugated rabbit anti-mouse anti-Ig (Sigma). After a further hour's agitation, this solution was discarded and unbound sera was removed by washing six times in 1x TBS as described above. Bound antisera was detected by the addition of 10 ml of a developing solution (see below). The reaction was allowed to proceed until bands were clearly visible, and the reaction was terminated by extensive rinsing of the membrane with dH_2O. The PVDF membrane was then allowed to air dry at room temperature.

Developing Solution:

1 ml of 0.1% (w/v) nitroblue tetrazolium in 1x TBS, was mixed with 0.1 ml of a solution of 5-bromo-4-chloro-3-indoyl phosphate (p-toluidine salt) in dimethyl formamide (5 mg ml^{-1}) and 9 ml of 100 mM Tris.Cl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$.

2.13 Southern analysis of genomic DNA from filamentous fungi

2.13.1 Preparation of colony blot for probe hybridisation

Whole colony blots were used to find clones carrying the successfully ligated whole synthetic apoaequorin gene.

Colonies were patched onto gridded replica LB agar plates with the appropriate selection. One of the plates was overlaid with an Amersham Hybond-N membrane and one without. 60 colonies were patched per plate, and the plates numbered and orientated. Plates were incubated as usual at 37 °C overnight.

To at least one of the membranes, a very small amount of positive and negative control DNA (insert DNA (aeqDA-14) which is used to make the probe, and empty plasmid, pGEM3z) was placed in empty grids and left to dry. The membranes were lifted from the plates and prepared by placing on Whatman 3MM paper soaked with the following solutions in the order given:

a) 0.5 M NaOH, 1.5 M NaCl (denaturing) for 7 min

b) 1 M Tris.Cl, pH 7.4 (neutralising) for 5 min

c) 1.5 M NaCl, 0.5 M Tris.Cl, pH 7.4 (binding DNA to membrane) twice for 3 min

The cell debris was removed from the membranes by gently washing in 2x SSC (see section 2.13.5), and wiping the membrane with a tissue. The DNA was UV cross linked by exposing the air-dried membranes, DNA side up to UV irradiation at a total energy dose of 0.4 J/cm^2 (λ= 312).

Pre-hybridisation of the membranes was carried out for 2 hours as described in section 2.13.6. The probe was prepared as described in section 2.14, and hybridisation carried out for 2 hours at 65 °C. The washing step used a stringent wash solution of 2x SSC, 0.1% SDS for 20 min at 65 °C and was repeated three times. The membranes were placed in Saranwrap and autoradiography carried out as described in section 2.15.

2.13.2 DNA extraction from Aspergillus

Ground mycelia were prepared from shaking liquid cultures as described in section 2.12.1.2. Aliquots of 0.3 g of mycelia were placed in Eppendorf tubes then 0.5 ml extraction buffer (see Table 2.19), 0.25 ml equilibrated phenol (section 2.3.4.2) and 0.25 ml chloroform were added.

Table 2.19: Extraction buffer

p-aminosalicylic acid (PAS)	4.8 g in 40 ml dH_2O
triisopropylnaphthalenesulphonic acid (TNS, Fisher Chemicals, UK)	0.8 g in 40 ml dH_2O
5x RNB (1 M Tris.Cl pH 8.5, 1.25 M NaCl, 0.25 M EDTA)	20 ml

Solutions made up fresh on day of use. PAS solution was added to the TNS solution whilst stirring, then the RNB added. A precipitate forms, which is left, and the supernatant is used as the extraction buffer.

The samples were vortexed for 10-20 sec and then centrifuged at 13,500 rpm in a microcentrifuge. The aqueous phase (upper layer) was removed to a clean Eppendorf tube, and extracted twice with 0.5 volumes phenol and 0.5 volumes chloroform. Samples were vortexed and centrifuged as previously described to separate the phenolic and aqueous layers. EtOH precipitation was then carried out on the supernatant by adding 0.1 volume 3 M Na-acetate, pH 5.6 and 2.5 volumes EtOH and incubating at -70 °C for 30 min. The precipitated nucleic acids were pelleted by centrifuging for 20 min at 20,000 g in a microcentrifuge. The pellet was washed with 70% EtOH, centrifuged again, air-dried and finally resuspended in 150 µl TE.

To remove RNA, 3 µl 20 mg/ml Rnase A was added to the nucleic acid preparation and the reaction incubated at 37 °C for 30 min. The DNA was purified using phenol:chloroform extraction as described above and in section 2.3.4.2.

2.13.3 DNA extraction from N. crassa

Still liquid cultures of individual transformants were grown up (section 2.2.2.3.2.4). Culture contents were poured onto two layers of sterile muslin supported over a 1 l plastic beaker to separate the mycelium from the growth medium. The mycelium was washed in 300 ml of dH_2O and excess water was removed by squeezing the mycelium against the cheese cloth with a pair of sterile forceps. The mycelium was then added to a mortar which had been pre-cooled and filled with liquid nitrogen.

The mycelium was ground with liquid nitrogen and transferred to a 50 ml polypropylene tube containing 15 ml of DNA extraction buffer (10 mM EDTA, 100 mM NaCl, 0.5% SDS, 10 mM β-mercaptoethanol, 40 mM Tris.Cl, pH 8.0). The tube was vortexed for 30 sec, snap-frozen in liquid nitrogen and stored at -70 °C. Samples were thawed rapidly in a 65 °C water bath to minimise exposure to nucleases and immediately 1 volume of water saturated phenol (equilibrated to pH 7.4 with 100 mM Tris.Cl, pH 7.4) was added. The tube was shaken well and centrifuged at 6000 g for 3 min, the aqueous phase was removed to a clean tube and a second phenol extraction performed. This was followed by a single chloroform extraction to remove contaminating phenol. The DNA was precipitated with 10% v:v 3 M NaAc, pH 5.5, and two volumes 100% EtOH. The DNA was recovered by pelleting it by centrifugation at 6000 g for 3 min. After desiccation the DNA pellet was redissolved in 500 µl TE (10 mM Tris.Cl, pH 7.6, 1 mM EDTA pH 8.0). A 250 µl aliquot of the sample was then incubated with 2 µl of RNase (10 U/ µl) at 37 °C for 30 min; this was followed by the phenol:chloroform extraction procedure described in section 2.3.4.2 to remove contaminating protein. An UV wavelength scan was performed to estimate the concentration and purity of the genomic DNA and gel electrophoresis (section 2.4) was used to examine its integrity.

2.13.4 Restriction enzyme digests of genomic DNA

Genomic DNA (10 µg) was digested with 15 U of the appropriate restriction enzyme and 1x buffer in a final volume of 20 µl in dH_2O. A 2 µl aliquot of the reaction was examined by gel electrophoresis for complete DNA digestion. For Southern analysis, samples were resolved in large gels (20 cm x 20 cm x 0.6 cm) of 0.8% agarose in 1 x TAE. The gels were electrophoresed at 2-3 V per cm overnight.

2.13.5 Transfer of DNA to nylon membranes

The method of capillary transfer of DNA to a solid support was based on the method developed by (Southern, 1975). Following electrophoresis the gel was photographed

under UV illumination. The gel was then inverted and soaked in 800 ml 0.25 M HCl for 10 min shaking at approx. 30 rpm. This treatment of brief depurination nicks DNA strands thereby enhancing the transfer of large fragments to the membrane. The 0.25 M HCl solution was removed and the gel rinsed briefly in dH_2O. Following this the gel was soaked in 800 ml of denaturation solution (1.5 M NaCl, 0.5 N NaOH) for 45 min shaking as before, to denature the DNA *in situ*. The solution was removed, the gel rinsed briefly in dH_2O and placed in 800 ml of neutralisation solution (1 M Tris.Cl, pH 7.4, 1.5 M NaCl) for 30 min with gentle agitation.

The blot was constructed as follows. A piece of Whatman 3MM paper was wrapped around a perspex support and placed inside a larger plastic container. The container was filled with 10 x SSC (20 x SSC is 175.3 g NaCl, 88.2 g Na.citrate in 1 l, adjusted to pH 7.0) to a depth of 2 cm and the 3 MM paper was allowed to wet by capillary action. Any air bubbles were removed using a glass rod. A sheet of Hybond™ N nylon membrane (Amersham, UK) was cut so that it was slightly larger than the gel and immersed in a dish of dH_2O for 5 min, prior to being soaked in 10 x SSC. The inverted gel was placed on the support and any air bubbles between it and the 3 MM paper were removed using a glass rod; overhanging sheets of parafilm were then placed on the gel edges to prevent the blot from short-circuiting. The nylon membrane was placed on top on the gel, with its edge aligned with the top of the gel or the wells if the portion of the gel above the wells had been removed. As before, any air bubbles were removed. Two pieces of 10 x SSC wetted 3MM paper were placed on top of the membrane, and air bubbles removed. An 8 cm stack of dry paper towels surmounted by a glass plate was placed on top and the whole assembly was weighed down with a 500 g weight. Blotting was allowed to proceed for between 4 and 16 h after which the blot was dismantled, the position of the gel wells marked on the filter with a pencil, and the filter rinsed briefly in 2 x SSC. The filter was allowed to air-dry and the DNA was fixed by exposing the filter DNA side up to UV irradiation at a total energy dose of 0.4 J/cm^2 (λ= 312).

2.13.6 Probe hybridisation

Hybridisation of a radioactive probe to the immobilised DNA followed the procedure described in section 2.14.

2.13.7 Washing hybridised filters of immobilised DNA

Following hybridisation the probe solution was removed and stored at -20°C. The filter was then washed in the hybridisation bottle using a series of washes going from low to high stringency:

a) 2 x 100 ml 4 x SSC +1% SDS for 30 min at 65 °C

b) 2 x 200 ml 2 x SSC +0.5% SDS for 30 min at room temperature

c) 1 x 200 ml 1 x SSC for 30 min at room temperature

After washing, the filter was air-dried and sealed in Saranwrap. Autoradiography was performed as described in section 2.15.

2.14 Radioactive probing of immobilised nucleic acids

Handling and disposal of radioactive isotopes was according to the regulations and advice of the Radiation Committee of the University of Edinburgh.

2.14.1 Probe synthesis using the random primer labelling method

The protocol used was based on the method for radiolabelling DNA restriction endonuclease fragments to high specific activity (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984). Approximately 200 ng of DNA was diluted with dH_2O so that the final volume of the reaction would be 30 µl, and boiled for 5 min to denature the DNA. Six µl of 5 x oligonucleotide-labelling buffer (oliB) (250 mM Tris.Cl, pH 8.0, 25 mM $MgCl_2$, 5 mM β-mercaptoethanol, 2 mM of dATP, dTTP,

dGTP, 1 M HEPES pH 6.6, 1 mg/ml random hexamers) was then added followed by 0.5 µl of 20 mg/ml BSA, 1.5 µl of Klenow (5 U/µl) and finally 30 µCi (3 µl) of [α^{32}P] dCTP (3 Ci µmole^{-1}). The reaction was incubated at 37 °C for 1 hour and stored at -20 °C until use.

2.14.2 Probe purification

The radioactive probe was purified by running the reaction mix through a column of G-50 Sephadex (Pharmacia, UK), which retains the unincorporated nucleotides but allows the newly synthesised probe to pass through. The column was set up in a 1 ml syringe barrel with a small piece of glass paper to retain the Sephadex at the bottom. TE, pH 7.6, was added to the column using a Pasteur pipette until it passed through the paper. A slurry of G50-sephadex in TE was added to the syringe barrel until it was 0.5 cm from the top. TE was then added to stop the column from running dry. The column was fixed in a clamp stand. The radioactive probe mix was loaded into the top of the column followed by the continued addition of TE. The two fractions of the column were monitored using a Geiger counter. The highly radioactive probe fraction (approximately 500 µl) was collected in an Eppendorf tube.

2.14.3 Hybridisation of the probe to immobilised nucleic acids

Hybridisation was carried out in a Hybaid HB-OV-BL hybridisation bottle (Hybaid). Filters were prehybridised to prevent non-specific binding in 35 ml of hybridisation buffer (4 x SSC, 20 mM Tris.Cl, pH 7.5, 10% dextran sulphate, 2 x Dehardts solution) (Denhardt's solution contains 1% polyvinylpyrrolidone, 1% bovine serum albumin, and 1% Ficoll in dH_2O) for 1 h at 65 °C. The probe was denatured by boiling for 5 min and then added to 15 ml of hybridisation buffer. This immediately replaced the pre-hybridisation solution and the filter and probe were incubated together overnight rotating at 65 °C in a Hybaid dual hybridisation oven (Hybaid).

2.15 Autoradiography

The probed filter sealed in Saranwrap was placed in a light-tight developing cassette with an image intensifying screen (Dupont, USA). A piece of Dupont Cronex 4 medical X-ray film (Dupont) was placed on top of the filter and exposed (at -70 °C for ^{32}P, and at room temperature for ^{35}S) for between 4 and 24 hours depending upon the level of radioactivity, to determine the optimal exposure time. Following exposure the film was processed using an automated X-ray developer.

2.16 Measurement of aequorin luminescence

2.16.1 Luminometer

Luminometry was performed using an EG & G Berthold LB96P Microlumat luminometer which was controlled from a dedicated PC. The luminometer was designed to measure bio- or chemiluminescence using 96 well microplates. Two built-in injectors allowed stimulation of samples, and the software which was supplied with the luminometer allowed all measurements to be programmed. The luminometer was calibrated to the optimal (knee) working voltage of 1496 V. The 96 well plates used were obtained from EG & G Berthold, and were white, with flat-bottomed wells.

2.16.2 Coelenterazine

Coelenterazine was purchased from Cambridge Bioscience (UK) or Biosynth AG (Switzerland). Manipulation of coelenterazine was performed in near-darkness. Large quantities were separated into smaller aliquots by dissolving in cold 100% MeOH dispensing the solution into Eppendorf tubes and dried down under vacuum. Tubes were wrapped in aluminium foil and stored at -70 °C. Coelenterazine was dissolved in 100% MeOH prior to use.

2.16.3 in vivo *aequorin constitution*

For *in vivo* luminometry of *Aspergillus* cultures, fungal growth and aequorin constitution were performed as described in section 2.2.1.2.3.3. Luminometry of samples was performed using suitable programs, following the manufacturer's recommendations.

To test whether the apoaequorin:His-tag fusion protein was active, *in vivo* luminometry of *E. coli* cultures carrying the His-tagged apoaequorin gene was carried out. Cultures were grown in 5 ml LB with the appropriate selection at 37 °C (section 2.2.3.4.2) overnight. Over-expression was induced (section 2.12.1.1) for 3 hours, then 100 µl of culture was added to wells of a multiwell plate. Coelenterazine was added to a final concentration of 2.5 µM, and the plate incubated at 37 °C for 4 hours. Luminometry of the samples was carried out as described above.

2.16.4 in vitro *constitution and luminometry of aequorin from protein extracts*

Protein extracts from *Aspergillus* and *N. crassa* (section 2.12.1.2), or purified His-tag apoaequorin from *E. coli* (section 2.12.5) were quantified (section 2.12.2) and 10 µg protein (unless otherwise stated) added to wells of a multiwell plate. The volume in each well was adjusted to 50 µl with extraction buffer (section 2.12.1.2). Coelenterazine was added to extraction buffer so that the final concentration was 2.5 µmoles per 50 µl. 50 µl of this solution was then added to each well, and the plate incubated in darkness, at room temperature for 4 hours. Luminometry of the samples was then performed using a suitable program, following the manufacturer's recommendations.

3. PRODUCTION OF A SYNTHETIC APOAEQUORIN GENE

3.1 Introduction

The initial aim of this work was to produce a level of apoaequorin expression higher than had previously been achieved in *N. crassa* (Collis, 1996). In the previous study it was concluded that the two main factors responsible for the low levels of apoaequorin expression were probably codon usage and protein degradation.

Codon usage is well described, and all organisms studied have a preferred set of codons (Wada *et al.*, 1992). Work in *E. coli* first showed that codon use is linked to tRNA levels, and that there is a greater bias in genes that are highly expressed compared with genes expressed at a low level (Ikemura, 1981). Similar results have been shown for *Saccharomyces cerevisiae* (Ikemura, 1985). In *E. coli*, measurements of the translation rate of mRNAs containing frequent codons or rare codons have shown that rare codons are translated six-fold slower than frequent codons (Sorensen *et al.*, 1989). Also in prokaryotes, differential codon usage has been found for genes that are expressed at different stages in the cell cycle (reviewed in (Saier, 1995)). Due to the kinetics of supply and demand of tRNA molecules, it has been shown that successive rare codons can slow translation due to a lack of the necessary tRNA. This has been shown to affect gene expression in *N. crassa* (Kinnaird *et al.*, 1991). In *E. coli*, similar observations have been reported (Chen and Inouye, 1990), with the effect being overcome by moving the rare codons further away from the initiation codon. A comparison of the native apoaequorin A gene (*aeqA*) with codon bias in *N. crassa* showed that *aeqA* contained 44 rare *N. crassa* codons, with 9 pairs of rare codons appearing successively as 'doublets' (Collis, 1996). This, together with data obtained on protein expression and mRNA levels

provided evidence for the hypothesis that codon bias may affect apoaequorin expression in *N. crassa* (Collis, 1996).

Therefore, the sequences of the two apoaequorin genes available were analysed and compared with the codon usage and recognition sequences of three fungi: *A. niger, A. nidulans* and *N. crassa,* with the view of possibly altering the gene sequence to optimise expression levels. The choice of producing a synthetic or semi-synthetic gene was investigated, and a suitable gene sequence designed. Production of a synthetic apoaequorin gene is described and the methods used to verify that the subsequent protein was functional.

3.2 Design of the synthetic apoaequorin gene, *gnaeqD*

3.2.1 Comparisons of codon bias amongst filamentous fungi

The codon usage in three fungal species (*A. niger, A. nidulans* and *N. crassa*) was analysed. Codon usage was calculated as a percentage of the use of each codon for each amino acid. A codon was designated rare if it was used less than 15% per amino acid. The number of genes used for each species are as shown in Table 3.1.

Table 3.1: Codon tables used in this study

Codon Usage Table	Species	No. of genes used for analysis	source/ reference
Table 3.2	*A. niger*	98	(Nakamura *et al.*, 1997)
Table 3.3	*N. crassa*	69	(Wada *et al.*, 1992)
Table 3.4	*A. nidulans*	45	(Wada *et al.*, 1992), (Lloyd and Sharp, 1991)

Table 3.2: Codon usage in *A. niger* (per amino acid)

	U		C		A		G		
U	Phe	23.6	Ser	17.7	Tyr	25.1	Cys	33.1	**U**
	Phe	**76.4**	**Ser**	**30.5**	**Tyr**	**74.9**	**Cys**	**66.9**	**C**
	Leu	1.3	Ser	5.3	---	---	---	---	**A**
	Leu	15.3	Ser	15.5	---	---	Trp	---	**G**
C	Leu	16.0	Pro	26.1	His	32.3	Arg	29.0	**U**
	Leu	30.9	**Pro**	**43.6**	**His**	**67.7**	**Arg**	**34.7**	**C**
	Leu	4.5	Pro	9.2	Gln	23.7	Arg	7.5	**A**
	Leu	**32.0**	Pro	21.1	**Gln**	**76.3**	Arg	13.6	**G**
A	Ile	28.8	Thr	22.2	Asn	23.6	Ser	9.5	**U**
	Ile	**68.0**	**Thr**	**52.4**	**Asn**	**76.4**	Ser	21.5	**C**
	Ile	3.2	Thr	9.2	Lys	13.7	Arg	7.6	**A**
	Met	---	Thr	16.2	**Lys**	**86.3**	Arg	7.6	**G**
G	Val	22.8	Ala	30.8	Asp	42.1	Gly	35.5	**U**
	Val	**45.0**	**Ala**	**42.7**	**Asp**	**57.9**	**Gly**	**40.0**	**C**
	Val	4.6	Ala	11.1	Glu	29.2	Gly	17.0	**A**
	Val	27.6	Ala	15.4	**Glu**	**70.8**	Gly	7.5	**G**

Key to Tables 3.1, 3.2 & 3.3: 1st column = first base, 1st row = second base, last column = third base in codon. Figures are percentages of use per amino acid. Amino acids and figures in bold represent the most popular codon for that amino acid.

Table 3.3: Codon usage in *N. crassa* (per amino acid)

	U		C		A		G		
U	Phe	22.2	Ser	16.2	Tyr	22.8	Cys	19.1	**U**
	Phe	**77.8**	**Ser**	**30.5**	**Tyr**	**77.2**	**Cys**	**80.9**	**C**
	Leu	1.6	Ser	6.5	---	---	---	---	**A**
	Leu	14.9	Ser	17.9	---	---	Trp	---	**G**
C	Leu	18.4	Pro	22.9	His	26.7	Arg	22.5	**U**
	Leu	**42.1**	**Pro**	**46.3**	**His**	**73.3**	**Arg**	**39.1**	**C**
	Leu	4.5	Pro	11.7	Gln	25.6	Arg	6.7	**A**
	Leu	18.3	Pro	19.1	**Gln**	**74.3**	Arg	9.6	**G**
A	Ile	31.4	Thr	19.6	Asn	17.7	Ser	7.1	**U**
	Ile	**63.4**	**Thr**	**53.9**	**Asn**	**82.3**	Ser	21.6	**C**
	Ile	5.1	Thr	10.7	Lys	10.4	Arg	8.2	**A**
	Met	---	Thr	15.7	**Lys**	**89.6**	Arg	13.9	**G**
G	Val	25.1	Ala	27.6	Asp	38.1	Gly	36.3	**U**
	Val	**54.6**	**Ala**	**51.8**	**Asp**	**61.9**	**Gly**	**45.5**	**C**
	Val	4.6	Ala	7.2	Glu	19.4	Gly	10.8	**A**
	Val	15.5	Ala	13.3	**Glu**	**80.6**	Gly	7.4	**G**

Table 3.4: Codon usage in *A. nidulans* (per amino acid)

	U		C		A		G		
U	Phe	28.1	Ser	20.4	Tyr	31.1	Cys	33.8	**U**
	Phe	**71.9**	**Ser**	**25.5**	**Tyr**	**68.9**	**Cys**	**66.2**	**C**
	Leu	5.7	Ser	13.6	---	---	---	---	**A**
	Leu	13.4	Ser	15.2	---	---	Trp	---	**G**
C	Leu	20.2	Pro	26.6	His	38.3	Arg	20.8	**U**
	Leu	**27.2**	**Pro**	**31.3**	**His**	**61.7**	**Arg**	**29.3**	**C**
	Leu	9.9	Pro	20.6	Gln	35.6	Arg	14.6	**A**
	Leu	23.6	Pro	21.3	**Gln**	**64.4**	Arg	16.3	**G**
A	Ile	35.1	Thr	25.7	Asn	29.2	Ser	8.8	**U**
	Ile	**56.1**	**Thr**	**35.5**	**Asn**	**70.8**	Ser	16.4	**C**
	Ile	8.8	Thr	21.5	Lys	27.4	Arg	9.6	**A**
	Met	---	Thr	17.3	**Lys**	**72.6**	Arg	9.4	**G**
G	Val	29.5	Ala	29.0	Asp	46.0	Gly	32.2	**U**
	Val	**42.4**	**Ala**	**36.4**	**Asp**	**54.0**	**Gly**	**35.1**	**C**
	Val	8.2	Ala	16.7	Glu	37.3	Gly	19.2	**A**
	Val	19.9	Ala	17.9	**Glu**	**62.7**	Gly	13.4	**G**

From comparisons of Tables 3.2-3.4, several conclusions can be drawn. All three fungi prefer the third base to be C, and use of a purine in the third position is very uncommon if there is a choice of a pyrimidine residue instead. This has been observed previously in *N. crassa* (Kinnaird *et al.*, 1991) and *A nidulans* (Lloyd and Sharp, 1991). The most popular codons in each species are seen to be identical, with the exception of the codon for leucine, which is CUG in *A. niger*. However, its frequency of use is almost identical to that of the second most popular codon (CUC), which is the most popular in both *A. nidulans* and *N. crassa*. The rare codons are similar in the three fungi analysed. Therefore, if a synthetic apoaequorin gene was designed, it should be possible to produce one sequence which will be equally usable for each species.

It is noticeable that the species with the most codon bias is *N. crassa*, then *A. niger*, and lastly *A. nidulans*. If codon bias is a factor which contributes to the low expression levels in *N. crassa*, then expression of native apoaequorin could be expected to be better in the *Aspergillus* species.

3.2.2 Comparison of codon usage in native apoaequorin genes with fungal codon usage

To determine the frequency of rare fungal codons in the native apoaequorin genes, the codon usage of apoaequorin was analysed. The two full cDNA sequences of apoaequorin available are called apoaequorin A (*aeqA*) and apoaequorin D (*aeqD*). Total codon usage in the two genes (Inouye *et al.*, 1985; Prasher *et al.*, 1985) is shown in Table 3.5. Both sequences are 197 amino acids in length. There are 54 nucleotide differences between *aeqA* and *aeqD*, and the codons which they affect mostly produce the same amino acid in both proteins. This is to be expected, as they are isoforms of one another, and both proteins show the same properties (Cormier *et al.*, 1989; Ohmiya and Tsuji, 1993). However, comparisons of incomplete cDNAs of other apoaequorin isoforms showed a higher level of heterogeneity than is seen

between *aeqA* and *aeqD* (Prasher *et al.*, 1987). In total, there are 19 amino acid differences between the two apoaequorin sequences.

Table 3.5: Codon usage in *aeqA* and *aeqD*

	U			C			A			G			
U	Phe	**2**	3	Ser	**2**	2	Tyr	**2**	2	Cys	**0**	0	**U**
	Phe	**6**	5	Ser	**0**	2	Tyr	**6**	6	Cys	**3**	3	**C**
	Leu	**1**	2	Ser	**4**	4	---	**1**	1	---	**0**	0	**A**
	Leu	**2**	2	Ser	**0**	1	---	**0**	0	Trp	**6**	6	**G**
C	Leu	**4**	5	Pro	**3**	3	His	**2**	1	Arg	**1**	2	**U**
	Leu	**3**	2	Pro	**1**	1	His	**3**	4	Arg	**0**	0	**C**
	Leu	**0**	0	Pro	**2**	2	Gln	**6**	6	Arg	**2**	1	**A**
	Leu	**2**	2	Pro	**0**	0	Gln	**1**	1	Arg	**0**	0	**G**
A	Ile	**5**	6	Thr	**2**	1	Asn	**4**	4	Ser	**1**	1	**U**
	Ile	**6**	6	Thr	**2**	3	Asn	**4**	4	Ser	**1**	1	**C**
	Ile	**1**	1	Thr	**6**	5	Lys	**12**	10	Arg	**3**	3	**A**
	Met	**5**	5	Thr	**1**	0	Lys	**4**	4	Arg	**0**	2	**G**
G	Val	**2**	2	Ala	**9**	7	Asp	**15**	13	Gly	**4**	3	**U**
	Val	**4**	3	Ala	**4**	2	Asp	**4**	5	Gly	**0**	1	**C**
	Val	**1**	2	Ala	**4**	3	Glu	**10**	13	Gly	**11**	11	**A**
	Val	**2**	1	Ala	**0**	1	Glu	**5**	4	Gly	**0**	0	**G**

Key: Figures in normal type are apoaequorin a, figures in bold are apoaequorin d. Figures indicate the number of times each codon is used throughout the sequences.

The codon usage of the two apoaequorin genes was then compared with the rare codons of the three fungal species (Tables 3.2-3.4), and their frequency of use recorded (Table 3.6).

Table 3.6: Fungal rare codon usage in native apoaequorin genes

amino acid	rare codons	*N. crassa*	*A. niger*	*A. nidulans*	*aeqA*	*aeqD*
Leu	CUA	4.5	5.2	9.9	0	0
	UUA	1.6	1.2	5.7	2	1
	UUG	14.9	---	13.4	2	2
Ile	AUA	5.1	---	8.8	1	1
Val	GUA	4.6	4.5	8.2	2	1
Ser	UCA	6.5	5.3	13.6	3	3
	AGU	7.1	8.7	8.8	1	1
	UCG	---	14.1	---	1	0
Pro	CCA	11.7	7.8	---	2	2
	CCG	---	---	---	0	0
Thr	ACA	10.7	8.7	---	5	5
	ACG	---	15	---	0	1
Ala	GCA	7.2	11.4	---	3	4
	GCG	13.3	14.6	---	1	0
Lys	AAA	10.4	14.5	---	10	11
Arg	CGA	6.7	7.9	14.6	1	2
	CGG	9.6	14.3	---	0	0
	AGA	8.2	7.4	9.6	3	3
	AGG	13.9	8.8	9.4	2	0
Gly	GGA	10.8	---	---	11	11
	GGG	7.4	7.5	13.4	0	0

Key: Numbers in columns for each organism indicate percentage usage per amino acid (codon usage > 15% not shown). Numbers in apoaequorin columns indicate total numbers of those codons in the apoaequorin genes.

As shown in Table 3.6, three or four of the rare fungal codons are used quite frequently in both *aeqA* and *aeqD*. These are: UCA (Ser), ACA (Thr), AAA (Lys) and GGA (Gly). Approximately 20% of the total codons in both native genes are rare in all three fungi. In the light of other studies on the effect of rare codons on gene expression, both *aeqA* and *aeqD* sequences were investigated to ascertain the positions of the rare codons. For this comparison, the codon bias of *A. niger* was used. The results are shown in Figures 3.1 and 3.2.

Figure 3.1: Native *aeqA* with rare codons of *A. niger* shown in bold text

5'	ATG	ACC	AGC	GAA	CAA	TAC	**TCA**	GTC	AAG	CTT	**ACA**
CCA	GAC	TTC	GAC	AAC	**CCA**	**AAA**	TGG	ATT	GGA	**CGA**	CAC
AAG	CAC	ATG	TTT	AAT	TTT	CTT	GAT	GTC	AAC	CAC	AAT
GGA	**AGG**	ATC	TCT	CTT	GAC	GAG	ATG	GTC	TAC	AAG	**GCG**
TCC	GAT	ATT	GTT	**ATA**	AAC	AAT	CTT	GGA	**GCA**	**ACA**	CCT
GAA	CAA	GCC	**AAA**	CGT	CAC	**AAA**	GAT	GCT	**GTA**	GAA	GCC
TTC	TTC	GGA	GGA	GCT	GGA	ATG	**AAA**	TAT	GGT	**GTA**	GAA
ACT	GAA	TGG	CCT	GAA	TAC	ATC	GAA	GGA	TGG	**AAA**	**AGA**
CTG	GCT	TCC	GAG	GAA	TTG	**AAA**	**AGG**	TAT	**TCA**	**AAA**	AAC
CAA	ATC	**ACA**	CTT	ATT	CGT	**TTA**	TGG	GGT	GAT	**GCA**	TTG
TTC	GAT	ATC	ATT	GAC	**AAA**	GAC	CAA	AAT	GGA	GCT	ATT
TCA	CTG	GAT	GAA	TGG	**AAA**	**GCA**	TAC	ACC	**AAA**	TCT	GAT
GGC	ATC	ATC	CAA	**TCG**	**TCA**	GAA	GAT	TGC	GAG	GAA	**ACA**
TTC	**AGA**	GTG	TGC	GAT	ATT	GAT	GAA	**AGT**	GGA	CAG	CTC
GAT	GTT	GAT	GAG	ATG	**ACA**	**AGA**	CAA	CAT	**TTA**	GGA	TTT
TGG	TAC	ACC	ATG	GAT	CCT	GCT	TGC	GAA	AAG	CTC	TAC
GGT	GGA	GCT	GTC	CCC	TAA	3'					

Figure 3.2: Native *aeqD* with rare codons of *A. niger* shown in bold text

5'	ATG	**ACA**	AGC	**AAA**	CAA	TAC	**TCA**	GTC	AAG	CTT	**ACA**
TCA	GAC	TTC	GAC	AAC	**CCA**	**AGA**	TGG	ATT	GGA	**CGA**	CAC
AAG	CAT	ATG	TTC	AAT	TTC	CTT	GAT	GTC	AAC	CAC	AAT
GGA	**AAA**	ATC	TCT	CTT	GAC	GAG	ATG	GTC	TAC	AAG	**GCA**
TCT	GAT	ATT	GTC	ATC	AAT	AAC	CTT	GGA	**GCA**	**ACA**	CCT
GAG	CAA	GCC	**AAA**	**CGA**	CAC	**AAA**	GAT	GCT	**GTA**	GAA	GCC
TTC	TTC	GGA	GGA	GCT	GGA	ATG	**AAA**	TAT	GGT	GTG	GAA
ACT	GAT	TGG	CCT	**GCA**	TAT	ATT	GAA	GGA	TGG	**AAA**	**AAA**
TTG	GCT	ACT	GAT	GAA	TTG	GAG	**AAA**	TAC	GCC	**AAA**	AAC
GAA	**CCA**	**ACG**	CTC	ATC	CGT	**ATA**	TGG	GGT	GAT	GCT	TTG
TTT	GAT	ATC	GTT	GAC	**AAA**	GAT	CAA	AAT	GGA	GCC	ATT
ACA	CTG	GAT	GAA	TGG	**AAA**	**GCA**	TAC	ACC	**AAA**	GCT	GCT
GGT	ATC	ATC	CAA	**TCA**	**TCA**	GAA	GAT	TGC	GAG	GAA	**ACA**
TTC	**AGA**	GTG	TGC	GAT	ATT	GAT	GAA	**AGT**	GGA	CAA	CTC
GAT	GTT	GAT	GAG	ATG	**ACA**	**AGA**	CAA	CAT	**TTA**	GGA	TTT
TGG	TAC	ACC	ATG	GAT	CCT	GCT	TGC	GAA	AAG	CTC	TAC
GGT	GGA	GCT	GTC	CCC	TAA	3'					

As can be seen in the these figures, both *aeqA* and *aeqD* contain 9 doublets (pairs of rare codons) of *A. niger* rare codons, and in *aeqD*, three of the first 10 amino acids

are rare. It is possible that the presence of 9 doublets may affect translation as hypothesised by Kinnaird *et al.* (1991). Other factors which may affect the translation rate are the proximity of rare codons to the initiation site, which has been shown to affect translation in *E. coli* (Chen and Inouye, 1990), and three of the first 10 codons in *aeqD* being rare.

Neither of the apoaequorin genes appears more biased than the other with regard to the codon usage of these fungi. The only possibly significant difference is the frequency of rare codons near the initiation site in *aeqD* being higher than that in *aeqA*. The high frequency of rare codons in both genes make the options for producing a synthetic gene limited. To remove all rare codons individually using site-directed mutagenesis would be tedious with either gene. The most plausible option is to completely design a synthetic gene. Choice of which amino acid sequence to use as the template theoretically does not matter, as both proteins show the same characteristics. The latter method was chosen, using the amino acid sequence of AEQD (Inouye *et al.*, 1985).

3.2.3 Development of a synthetic apoaequorin gene for Aspergillus

Since it was decided to produce a completely synthetic apoaequorin gene to overcome the presence of rare codons, the whole sequence was altered to use the most popular codons of *A. niger*. Unique restriction endonuclease sites were introduced to ease cloning of the gene. Also, unique restriction sites were placed within the gene's sequence to allow for production of the gene as two polynucleotides which could then be pieced together. All alterations of the gene sequence were made with the codon usage of *A. niger* in mind, and without altering the amino acid sequence.

The nucleotide sequence of synthetic apoaequorin D (*gnaeqD*) was checked for the presence of possible target sites which would affect the expression of the final mature protein in filamentous fungi. Also, the amino acid sequence of *aeqD* was examined for the presence of post-translational recognition sites. Table 3.7 below shows the type of target sites searched for and the sequences involved.

Table 3.7: Post-transcriptional target sites of apoaequorin D mRNA and protein

Target site	Sequences searched for	Results
At nucleotide level:		
5' splice site	5' GTNNGT 3'	none found
internal lariat sequence	5' PyGCTPuACN 3'	base 32-9; AGCTTACC[1]
3' splice site	5' PyAG 3'	base 187-9, nearest to lariat site[2]
At protein level:		
O-linked glycosylation	Ser or Thr plus target sequence[3]	none found
N-linked glycosylation	Asn-X-Ser/Thr	none found
mitochondrial entry sequence	positively charged amino acids with Ser & Thr[4]	none found
secretory signal	Arg/Lys & run of hydrophobic amino acids	amino acids 66, then 71-78
ubiquitin targeted degradation	Lys plus degradation sequence[5]	none found
	amino terminal residue type	Thr -resistant[6]

Key: [1] Putative lariat site is not exactly the same as the consensus sequence searched for.
[2] 3' splice site is too far from the putative lariat formation site for either to be of significance.
[3] Target sequences appear to vary and are generally long and complex. Therefore, the result 'none found' in this instance should be interpreted as 'no target sequence known in the gene'.
[4] The searched for sequence is based upon a general eukaryotic consensus sequence, and not on anything specific to filamentous fungi.
[5] The degradation sequence for filamentous fungi is not known, and therefore the result is not very certain.
[6] Eukaryotic studies show that the amino terminal amino acid is important in ubiquitin targeted degradation. Threonine is one which is found on proteins with long half lives (Bachmair *et al.*, 1986; Reiss *et al.*, 1988).

Although a sequence similar to the lariat consensus sequence was found at the nucleotide level, there was no 5' splice site, and the 3' splice site was too far from the lariat sequence. The only possible post-translational signal sequence uncovered

was that of a secretory signal. It was not as large as would normally be seen, and the consensus sequence searched for is for eukaryotes in general and not for filamentous fungi specifically. It cannot realistically be altered in the protein sequence as the effect of changing it upon the activity and structure of the protein is unknown. The lysine residue at position 66 could be changed, but there is another lysine and an arginine in close proximity (Figure 3.4). The final synthetic sequence was designed as shown in Figure 3.3. Since only the nucleotide sequence was changed, the amino acid sequence remained the same. The amino acid sequence for AEQD, as translated from both *aeqD* and *gnaeqD* is given in Figure 3.4.

Figure 3.3: Sequence of synthetic apoaequorin D gene, *gnaeqD*

CCGCAGACCT	GAATTC**ATG**A	CCTCCAAGCA	GTACTCCGTC	AAGCTTACCT	50
CCGACTTCGA	CAACCCCCGC	TGGATCGGCC	GCCACAAGCA	CATGTTCAAC	100
TTCCTCGACG	TCAACCACAA	CGGCAAGATT	TCCCTCGACG	AGATGGTCTA	150
CAAGGCCTCC	GACATCGTCA	TCAACAACCT	CGGCGCTACC	CCCGAGCAGG	200
CCAAGCGCCA	CAAGGACGCC	GTCGAGGCCT	TCTTCGGCGG	TGCCGGCATG	250
AAGTACGGCG	TCGAGACCGA	CTGGCCCGCC	TACATCGAGG	GCTGGAAGAA	300
GCTCGCCACC	GACGAGCTCG	AGAAGTACGC	CAAGAACGAG	CCCACCCTCA	350
TCCGCATCTG	GGGCGACGCC	CTCTTCGACA	TCGTCGACAA	GGACCAGAAC	400
GGTGCCATCA	CCCTCGACGA	GTGGAAGGCC	TACACCAAGG	CCGCCGGCAT	450
CATTCAGTCC	AGCGAGGACT	GCGAAGAGAC	CTTCCGCGTC	TGCGACATCG	500
ACGAGTCCGG	CCAGCTCGAT	GTCGATGAGA	TGACCCGCCA	GCACCTCGGC	550
TTCTGGTACA	CCATGGACCC	CGCCTGCGAG	AAGCTCTACG	GCGGTGCCGT	600
CCCCT**AA**GAT	CTAAGCTTGG	ATCCAGCCTG	A		631

Key: Start (ATG) and termination (TAA) codons shown in bold

Figure 3.4: Amino acid sequence of apoaequorin D (AEQD)

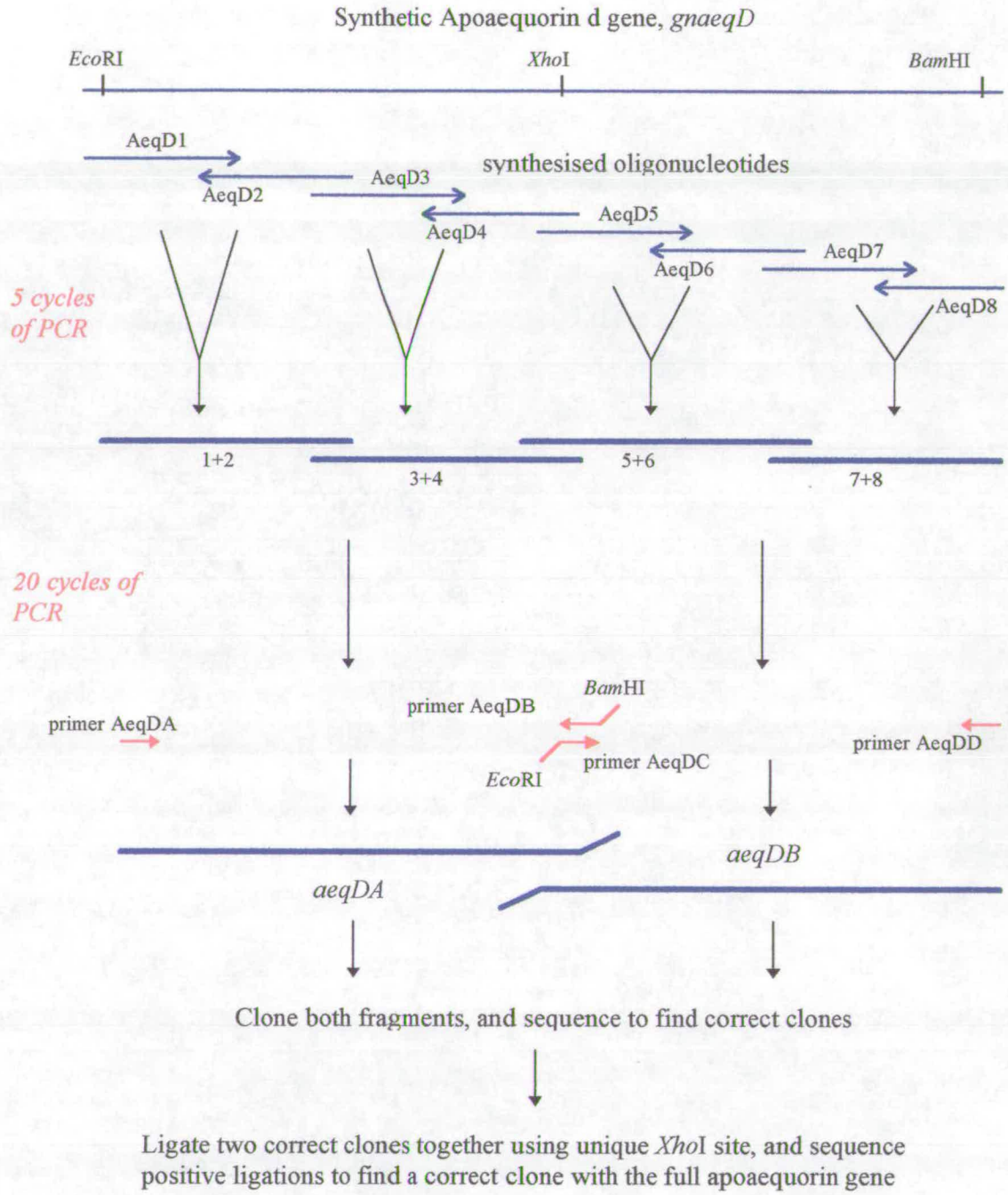
MET	THR	SER	LYS	GLN	TYR	SER	VAL	LYS	LEU	10
THR	SER	ASP	PHE	ASP	ASN	PRO	ARG	TRP	ILE	20
GLY	ARG	HIS	LYS	HIS	MET	PHE	ASN	PHE	LEU	30
ASP	VAL	ASN	HIS	ASN	GLY	LYS	ILE	SER	LEU	40
ASP	GLU	MET	VAL	TYR	LYS	ALA	SER	ASP	ILE	50
VAL	ILE	ASN	ASN	LEU	GLY	ALA	THR	PRO	GLU	60
GLN	ALA	LYS	ARG	HIS	LYS	ASP	ALA	VAL	GLU	70
ALA	PHE	PHE	GLY	GLY	ALA	GLY	MET	LYS	TYR	80
GLY	VAL	GLU	THR	ASP	TRP	PRO	ALA	TYR	ILE	90
GLU	GLY	TRP	LYS	LYS	LEU	ALA	THR	ASP	GLU	100
LEU	GLU	LYS	TYR	ALA	LYS	ASN	GLU	PRO	THR	110
LEU	ILE	ARG	ILE	TRP	GLY	ASP	ALA	LEU	PHE	120
ASP	ILE	VAL	ASP	LYS	ASP	GLN	ASN	GLY	ALA	130
ILE	THR	LEU	ASP	GLU	TRP	LYS	ALA	TYR	THR	140
LYS	ALA	ALA	GLY	ILE	ILE	GLN	SER	SER	GLU	150
ASP	CYS	GLU	GLU	THR	PHE	ARG	VAL	CYS	ASP	160
ILE	ASP	GLU	SER	GLY	GLN	LEU	ASP	VAL	ASP	170
GLU	MET	THR	ARG	GLN	HIS	LEU	GLY	PHE	TRP	180
TYR	THR	MET	ASP	PRO	ALA	CYS	GLU	LYS	LEU	190
TYR	GLY	GLY	ALA	VAL	PRO	STOP				196

3.3 Gene synthesis and sequencing

3.3.1 Strategy for gene synthesis

An overview of the planned method is shown in Figure 3.5. Eight synthetic oligonucleotides (oligos) were designed (AeqD1- AeqD8) which overlapped to cover the whole gene (sequences shown in Table 2.1). Each oligo was 106 nucleotides long, except the last, AeqD8, which was 99 nucleotides long. They each overlapped with the adjacent oligos by 30 base pairs (bp). Formation of the whole gene was carried out in stages. Using PCR, the hybridised products of two adjacent oligos were amplified into a quarter of the gene (or a 'di-oligo'), relying on the 30 bp overlap to act as a primer in each direction. Two adjacent 'quarters' could then be amplified into a gene 'half' by mixing the products of the first round of PCR and selecting for PCR products of the annealing of the two quarters by adding primers for the furthest ends of the gene half. This was done with the four pairs of oligos to produce four 'di-oligo' fragments of the synthetic apoaequorin gene, which were then used to selectively PCR-amplify two gene halves. The separate halves, *aeqDA* and *aeqDB*, were then cloned into the T-vector, pTAg (R&D Systems, UK). Successfully ligated clones were selected using ampicillin and blue-white screening, and plasmid DNA purified from picked clones. Vectors containing an insert of the correct size and with the expected restriction fragment sizes were sequenced to find copies of both halves with the correct sequence.

Figure 3.5: Method of synthesis of synthetic apoaequorin D (*gnaeqD*) gene



Key: Sequences of synthetic oligos and PCR primers are given in Table 2.1 and Table 2.2 respectively.

The PCR conditions that were used for gene synthesis are given in Tables 3.8 and 3.9. The first gene half, *aeqDA*, was synthesised twice using PCR, as the sequenced clones from the first PCR were found to contain a high number of single base deletions and substitutions. The errors were found at different sites in each clone, and therefore were not mistakes in the synthetic oligos, but PCR-induced mutations. To try and overcome this problem, the second attempt was carried out using a mixture of *Taq* DNA polymerase and *Pfu* DNA polymerase, called Expand (Boehringer Mannheim, UK). *Pfu* is a 3'-5' proof-reading polymerase that can correct mis-matches in the PCR product as it is synthesised. Also, the annealing time was extended, although the temperature was lowered in an attempt to increase the yield. The clones from PCR 2 of *aeqDA* were not found to be any better for the rate of mutations than those obtained using *Taq* alone. Since the only way to accurately analyse the results of altering PCR parameters is to sequence the products, it would have required more work to optimise the PCR conditions than it would to search for correct clones. Therefore, the products from the initial PCR amplifications were used to search for correct sequences. PCR of the second gene half, *aeqDB*, was carried out using *Taq* (Table 3.9). Again, sequenced clones were found to contain a large number of mistakes.

Table 3.8: PCR conditions used for synthesis of the gene half, *aeqDA*

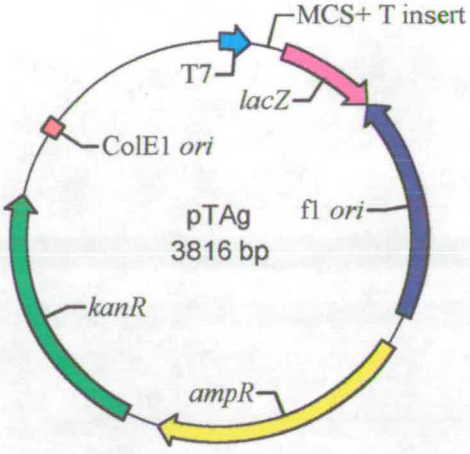
PCR 1:	**cycle**	**temp.**	**time**	**enzyme**
5 and 20 cycles	denature	94°C	1.1 min	
	annealing	73°C	0.5 min	
	polymerisation	73°C	4 min	
	final extension	73°C	7 min	*Taq* polymerase
PCR 2:				
5 and 20 cycles	denature	92°C	2 min	
	annealing	60°C	2 min	
	polymerisation	60°C	2.5 min	
	final extension	72°C	10 min	Expand

Table 3.9: PCR conditions used for synthesis of the gene half, *aeqDB*

PCR 1:	**cycle**	**temp.**	**time**	**enzyme**
5 cycles	denature	94°C	2 min	
	annealing	73°C	0.5 min	
	polymerisation	73°C	4 min	
	final extension	73°C	7 min	*Taq* polymerase
20 cycles	denature	94°C	2 min	
	annealing	62°C	2 min	
	polymerisation	73°C	4 min	
	final extension	73°C	7 min	*Taq* polymerase

Lowering the annealing temperature in the 20 cycles increased the yield of full length product, due to the lower temperature being optimal for the short primers (AeqDA, AeqDB, AeqDC and AeqDD) used in this PCR. The PCR products were analysed by agarose gel electrophoresis (one tenth of the samples were loaded), and found to contain smaller bands representing products from the di-oligos. This was expected as it was possible for these to be amplified in the 20 cycles as well as the complete gene halves. Also, there was slight 'smearing' of the bands, showing that there were products of various sizes produced. The PCR reactions were therefore purified by agarose gel electrophoresis. The correct sized bands were cut out and the DNA extracted using Qiagen QiaexII. Ligation of the recovered DNA into the T-vector pTAg (Figure 3.6) was carried out at different ratios of insert:vector to optimise the reaction. *E. coli* strain DH5α was then transformed with the ligation mixtures using heat shock, and cells plated out on blue/white screening plates (LB agar with ampicillin, IPTG and X-gal). Positive clones (white colonies) were picked and plasmid DNA purified from mini cultures using Promega Wizard mini-preps.

Figure 3.6: T-cloning vector, pTAg (R&D Systems, UK)



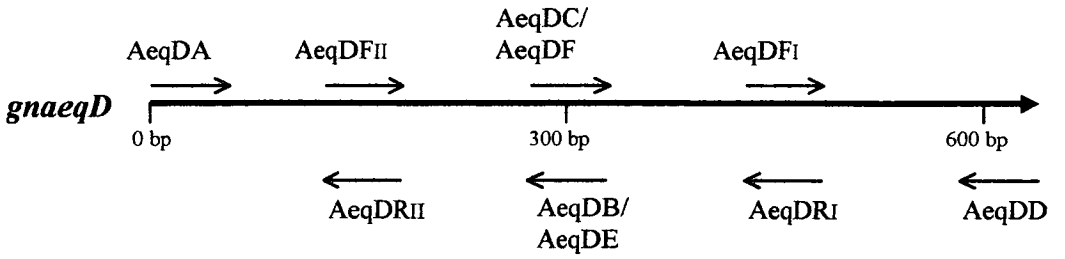
Key: Vector is shown circular in diagram for convenience. It is actually linear, due to the single T overhang. MCS; multiple cloning site. T insert; position of T overhang for cloning of PCR products. *lacZ*; β-galactosidase. *ampR*; ampicillin resistance. *kanR*; kanamycin resistance. T7; T7 promoter. F1 ori; F1 origin of replication. ColE1 ori; *E. coli* origin of replication.

Initial analysis of cloned PCR products was performed using restriction enzyme digests to ascertain whether the picked clones contained a fragment of the correct size and with internal restriction sites in the expected positions. Generally, for both *aeqDB* and *aeqDA* clones, a double digest using *Pst*I and *Xba*I was carried out to excise the insert from the multiple cloning site (MCS), and a double digest using *Eco*RI and *Xho*I used to cut inside the insert and in the MCS to ascertain the insert orientation. If the results were inconclusive, then further digests were used to analyse the insert. A large number of positive clones were obtained from all transformations of PCR products. Selected clones containing an insert of the correct size were then manually sequenced.

3.3.2 Sequencing PCR products

Initially, primers flanking the MCS of pTAg (pTAgSEQ5' and pTAgSEQ3', Table 2.2) were used for sequencing. However, due to the GC richness (62% GC) of *gnaeqD*, a lot of regions within the sequence are very susceptible to compressions, which compact the bands on the sequencing gel making it difficult to read the actual sequence. Using a primer near the GC rich regions can often overcome the compressions, and therefore primers within the gene halves were created (described in Table 2.2). The positions of these primers are shown in Figure 3.7.

Figure 3.7: Position of primers designed for *gnaeqD*



Also, sequencing was performed using normal nucleotides and using a guanidine analogue called inosine. Inosine creates weaker bonds within the sequence, which allows the oligonucleotides to migrate through the sequencing gel at their correct molecular weight. A disadvantage of using inosine is that the sequencing runs obtained are generally shorter and stops can occur, which make it impossible to decipher the identity of a particular base. Therefore, reactions using normal nucleotides were carried out at the same time as inosine sequencing reactions to compare the results.

In total, 21 clones containing *aeqDA* and 23 clones containing *aeqDB* were sequenced using guanidine and inosine nucleotides. From these, no completely correct clones were found. The types of errors found are summarised in Table 3.10.

Table 3.10: Errors found in clones of *aeqDA* and *aeqDB*

Type of error	No. of clones sequenced with error
1 bp deletion	21
2-10 bp deletion	10
11-20 bp deletion	5
single base substitution	1
single base insertion	1
multiple base insertion	2
incorrect annealing of oligos	8

Since all the clones investigated contained at least one error, it seemed very unlikely that a 100% successful PCR product would be found. From the sequences analysed, it was apparent that it should be possible to find two clones of each gene half which contained complementary correct sequences. It would then be possible to ligate these together using a unique restriction site in the overlapping region.

3.3.3 Formation of correct gene halves

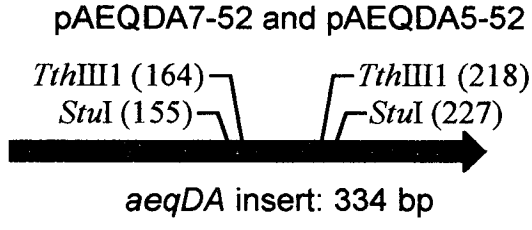
Analysis of the sequenced clones obtained showed two promising clones for both *aeqDA* and *aeqDB*. The clones, and their known mistakes are outlined in Table 3.11.

Table 3.11: PCR clones used for production of correct gene halves

clone	sequence errors
aeqDA (1-334 bp)	
pAEQDA7-52	ΔC 197
pAEQDA5-52	ΔC 235, G→C substitution 237, ΔG 301
aeqDB (305-631 bp)	
pAEQDB2-142	ΔATC 356-358
pAEQDB8-142	ΔC 463, ΔTCG 499-501

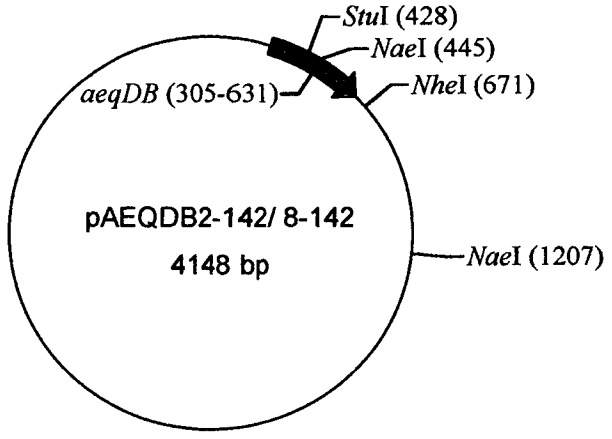
In addition to the errors shown in Table 3.10, the sequences of these four clones could not be assumed to be definitely correct due to the presence of compressions which had not been fully overcome using inosine for sequencing. The only way to surmount this problem was to use automated sequencing. Therefore, Qiagen maxi-preps were prepared for each clone to produce clean DNA suitable for automated sequencing. The 5' and 3' pTAg primers were used, as these should give a long enough sequence to give the whole insert on both strands. Automated sequencing results verified that the sequences were as determined using manual sequencing, with the exception of the G→C substitution in pAEQDA5-52, which was shown to be part of an unresolved compression. Suitable restriction sites were found in the four plasmids to allow for ligation of the complementary correct fragments. The results are shown in Figures 3.8 and 3.9.

Figure 3.8: Suitable restriction sites in *aeqDA*



Key: Figure shows the *aeqDA* insert in both pAEQDA7-52 and pAEQDA5-52. The restriction sites shown are the only ones present in *aeqDA*, and none are present in the host vector, pTAg.

Figure 3.9: Suitable restriction sites for ligation of two *aeqDB* clones



Key: Plasmid represents both pAEQDB2-142 and pAEQDB8-142. Both *aeqDB* clones are present in pTAg (Figure 3.6). Restriction sites shown are unique to plasmids, and are present in both clones.

As shown in Figure 3.8, either a 72 bp *Stu*I fragment or a 54 bp *Tth*III1 fragment can be isolated from pAEQDA5-52 and used to replace the corresponding fragment in pAEQDA7-42 which contains a single base deletion at 197 bp. Re-ligation of the fragment should be unidirectional, as the overhangs produced by the two enzymes are different.

Figure 3.9 shows that both pAEQDB2-142 and pAEQDB8-142 can be cut with *Nae*I to release a fragment of 762 bp. However, *Nae*I produces blunt-ended DNA molecules, and therefore re-ligation would be bidirectional. Digesting both plasmids with *Stu*I and *Nhe*I would release a smaller, 243 bp fragment containing the correct sequence in pAEQDB2-142 which can be used to replace the same fragment in pAEQDB8-142. The digestion overhangs produced by these two enzymes are not compatible (*Stu*I leaves blunt ends, and *Nhe*I leaves a CTAG overhang), and therefore ligation would be unidirectional. Therefore, the double digest was carried out on both the pAEQDB clones.

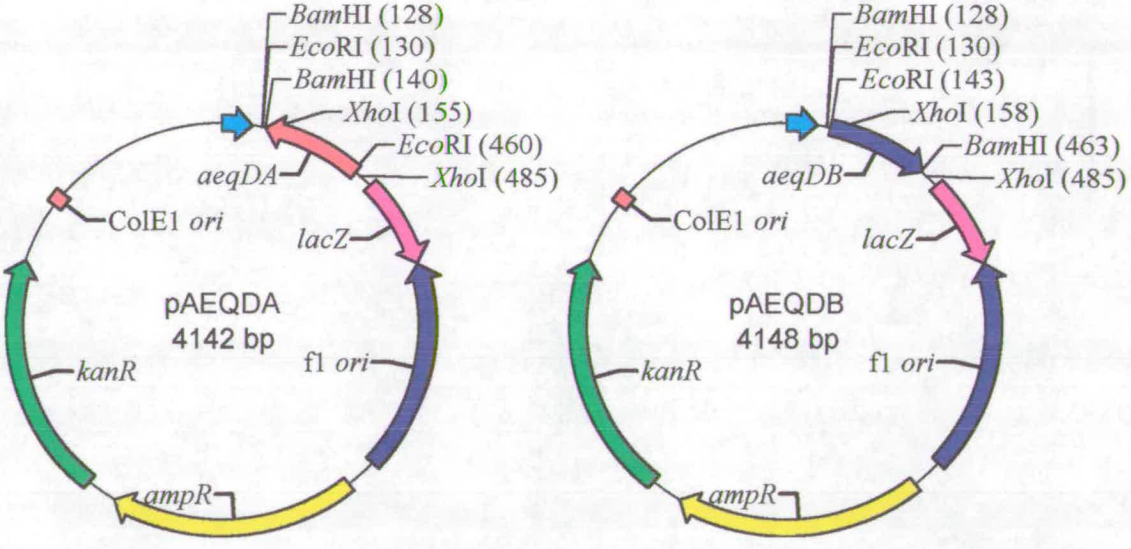
Both the possible methods (utilising *Stu*I or *Tth*III1) were carried out to increase the chance of success. Excised gel digest fragments were purified through silanized glass wool, precipitated and resuspended. Concentrations were estimated by agarose gel electrophoresis, and several ratios of vector:insert set up for ligation. The ratios used were 1:1, 1:3, 1:5, 2:1, 1:10 and 1:0 for vector:insert for both the *Tth*III1 and the *Stu*I digests, with 100-200 ng DNA in each ligation. Successful ligations were obtained with both digests, and clones were mini-prepped from both. These were checked by restriction digest for the presence of the correct sized insert. To determine if the insert was the correct one, clones were sequenced using the internal primers AeqDA and AeqDE. In total, 19 clones were sequenced, and of these, three had successfully inserted the correct sequence. These three clones (A5-7#13, 14 and 17) were completely sequenced using both guanidine and inosine nucleotides. From the sequencing results obtained, A5-7#14 and A5-7#17 were the most promising clones, as a compression in A5-7#13 could not be resolved. Therefore, clones 14 and 17 were used for producing the full length gene.

Production of a correct pAEQDB clone was carried out successfully using the *Stu*I/*Nhe*I double digest described above. However, transformation efficiency was very low using heat shock of DH5α, and therefore commercial XL-1 Blue chemically competent cells were used (Stratagene, UK). These cells have an efficiency of 5x 10^9, which is at least one magnitude higher than the transformation efficiency of DH5α cells. Enough clones were obtained with these cells, and after analysing mini-preps by restriction digest, eight out of twenty clones were found to contain the correct sized insert. Sequence analysis of these clones produced two, B2-8#10 and B2-8#12, which appeared to have correct sequence throughout the whole gene half, and these two clones were used for production of the complete gene.

3.3.4 Synthesis of the full gnaeqD *gene*

After the production of correct half-gene clones, a strategy was determined for producing the complete gene sequence. Initially, it was planned to cut both gene halves at the unique *Xho*I site in their overlapping region and at another unique site outside the gene coding sequence. Then one gene half would be ligated into the vector containing the other gene half. However, the cloning vector in which the PCR products had been cloned, pTAg, also contained an *Xho*I site. In theory it would have been possible to use the *Xho*I sites in a single digest and search for clones containing the correctly orientated insert. In practice, it was known by restriction analysis that both the *aeqDA* clones were inserted into pTAg anticlockwise, and both the *aeqDB* clones clockwise (see Figure 3.10). This makes it impossible to use the *Xho*I sites for construction of the gene unless a three-way ligation was used, which would be difficult to produce correctly re-ligated products.

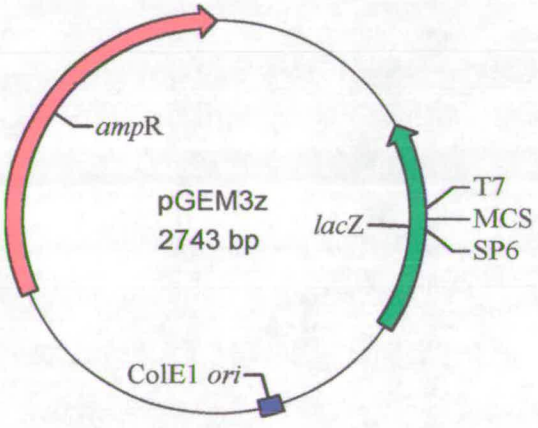
Figure 3.10: Orientation and selected restriction sites in *aeqDA* and *aeqDB* clones



Key: pAEQDA represents the clones A5-7#14 and A5-7#17. pAEQDB represents the clones B2-8#10 and B2-8#12. For information on the vector backbone, see Figure 3.6.

Other options for producing the full gene included using PCR to amplify up a full length product, using partial digests of the *Xho*I site or cloning one gene half into another vector and then adding the second half. PCR was ruled out after the problems encountered with the initial gene synthesis. Using a partial digest to linearise the plasmid and then selecting the linearised plasmid by agarose gel electrophoresis would be difficult to do, as it would be unknown which *Xho*I site had been cut, and would therefore entail screening a large number of clones by restriction analysis. Cloning one gene half into a different vector which contained no *Xho*I sites would probably be the simplest method. The chance of mutations occurring during excision and re-ligation is very slim, and therefore this would constitute little risk to the gene sequence.

Figure 3.11: Cloning vector pGEM3z (Promega, UK)



Key: MCS; multiple cloning site. *lacZ*; β-galactosidase. *ampR*; β-lactamase, ampicillin resistance. T7; T7 promoter. SP6; SP6 promoter. ColE1 ori; *E. coli* origin of replication.

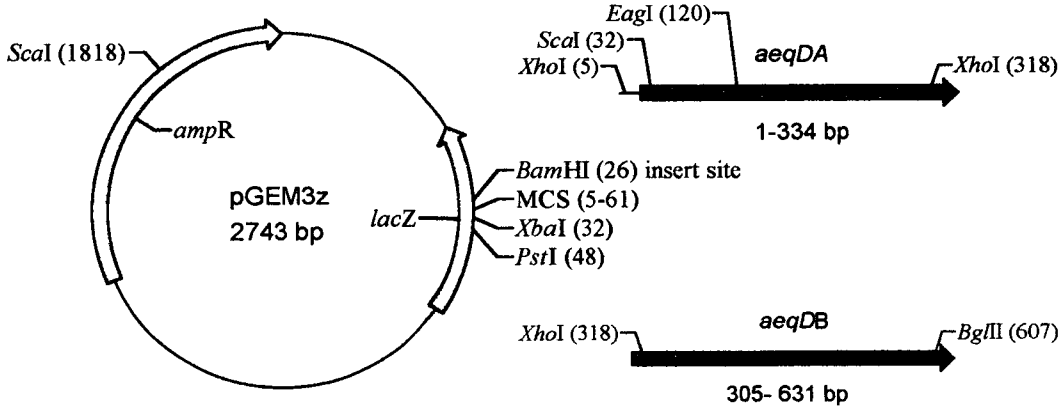
pGEM3z (Figure 3.11) is a cloning vector that contains no *Xho*I sites, and this was used as the backbone vector. Both *aeqDB* clones could be excised from pTAg with *Bam*HI, which cuts on both sides of the coding sequence (Figure 3.10), and inserted into the *Bam*HI site in the MCS of pGEM3z. This was done as described previously for fragment isolation and *E. coli* transformation, using ampicillin resistance and blue/white screening for selection. DH5α cells were used for transformation, and miniprepped clones were analysed by single restriction digests; using *Stu*I (cuts only in *aeqDB*) and *Bam*HI (excises the inserted *aeqDB* fragment). The successful

pGEM3z clones used were named as follows: clones produced using *aeqDB* from B2-8#10 were named B10-1 and B10-3; clones produced using *aeqDB* from B2-8#12 were named B12-1 and B12-2.

In an effort to avoid the possibility of one clone having an incorrect gene sequence, both *aeqDA* clones and the four named pGEM3z *aeqDB* clones were used for production of the whole gene. The *Xho*I site was used as planned. B10-1, B10-3, B12-1 and B12-2 were all digested with *Xho*I and the cut vectors gel-purified using QiaexII. Both the correct *aeqDA* clones, A5-7#14 and A5-7#17, were also digested with *Xho*I to excise the *aeqDA* fragment with a small piece of the MCS of pTAg at the 5' end of the gene sequence. The correct sized fragments (~350 bp) were gel purified as described for the *aeqDB* clones. Ligations were set up using all eight possible combinations plus the relevant controls, each at two ratios of insert:vector. Ligation was performed as usual at 16 °C overnight. Two aliquots (2 µl and 5 µl from a 20 µl reaction mix) of each ligation were used in transformation of DH5α cells. The control plates produced a very high background, although all transformations worked. This was probably due to very inefficient phosphatasing of the linearised vector. Since there were a large number of ligations and transformations, initially only two of the transformations were used for picking clones. These were the transformations of the ligation of B10-1 and A5-7#14, and the ligation of B12-1 and A5-7#14. Ten colonies were picked from each and miniprepped. Restriction analysis of the twenty plasmids showed them all to be religated vector. The options available to overcome the high level of background were to either screen a large number of the transformants or to redo the digestion, ligation and transformation. A large number of clones could be screened quite quickly using colony hybridisation with the *aeqDA* insert as a probe. This method was chosen, and 360 colonies from the transformation plates were replica-plated onto LB agar plates containing ampicillin. Positive and negative controls were also included. Colony hybridisation was carried out as described in Section 2.13.1, and autoradiography revealed 9 positive colonies. These putative whole gene clones

were picked and named pGNAEQD1 to pGNAEQD9. Miniprepped DNA from the nine clones was analysed by restriction analysis. Since the clones were produced from ligation of a single digest, it was possible that the orientation of the *aeqDA* insert was incorrect. Therefore, suitable restriction sites were found in the vector and both fragments of *gnaeqD* (Figure 3.12) to determine their orientations with respect to one another.

Figure 3.12: Restriction sites in pGEM3z, *aeqDA* and *aeqDB* used for analysis of full length clones



Key: For details of pGEM3z, see Figure 3.11. *Bam*HI is the insert site of *aeqDB*. The *Xho*I site in *aeqDB* was used to insert the *aeqDA Xho*I fragment (see text). Other sites shown were used to determine *aeqDA* and *aeqDB* orientation.

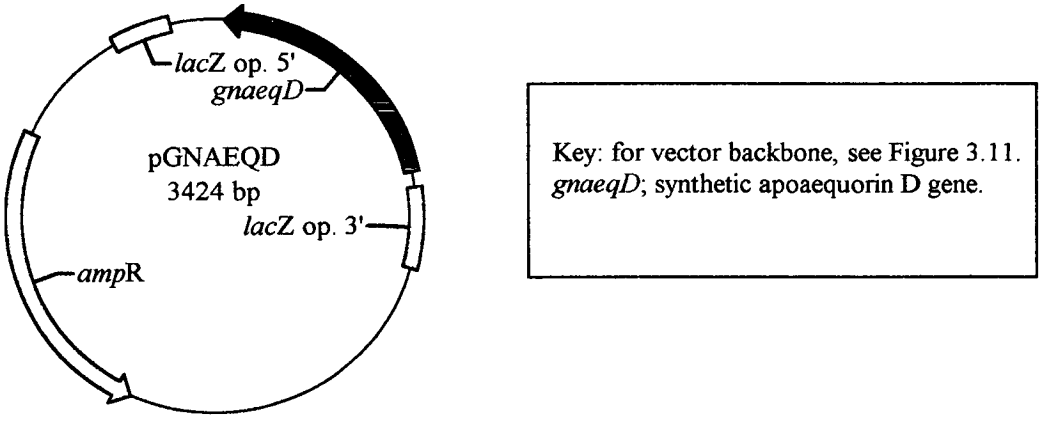
Table 3.12 shows the digests that were performed to verify the insertion of *aeqDA* and to determine the orientation of both gene fragments.

Table 3.12: Restriction digests used to analyse full length clones

Restriction digest	Expected results (from Figure 3.12)
*Xho*I	will excise *aeqDA*
*Xba*I and *Bgl*II	will show orientation of a*eqDB*
*Pst*I and *Bgl*II	will show orientation of *aeqDB*
*Sca*I	will show orientation of *aeqDA*
*Eag*I and *Pst*I	will show orientation of *aeqDA*

If digestion with *Xho*I produces a fragment of approx. 350 bp, this proves that *aeqDA* has been inserted without damage to either of the reformed *Xho*I sites. By combining the results obtained with the four other digests, it is possible to determine the orientation of both fragments. The results of this analysis showed that pGNAEQD2, pGNAEQD3 and pGNAEQD8 contained the full gene inserted correctly, anticlockwise (Figure 3.13). These three clones were then sequenced over the *Xho*I internal ligation site to verify that they contained no errors. The sequence was proven to be correct 150 bp in both directions from the ligation site. The whole gene in both pGNAEQD2 and pGNAEQD3 was then fully sequenced manually to verify that the remainder of the gene sequence was correct. A compression at approx. 600 bp in *gnaeqD* proved difficult to resolve in both clones, and a second compression at approx. 180 bp in pGNAEQD2 could not be resolved either. Therefore the two clones were automatically sequenced, using the primers T7, SP6, AeqDRI and AeqDFII. This proved that pGNAEQD3 contained the correct sequence for *gnaeqD*, but failed to resolve the second compression at 180 bp in pGNAEQD2 on one strand. Therefore pGNAEQD3 was chosen as the copy of *gnaeqD* to use in future applications, and a CsCl maxiprep of the plasmid was prepared.

Figure 3.13: pGNAEQD containing correctly inserted full length *gnaeqD*



3.4 Verification of *gnaeqD*

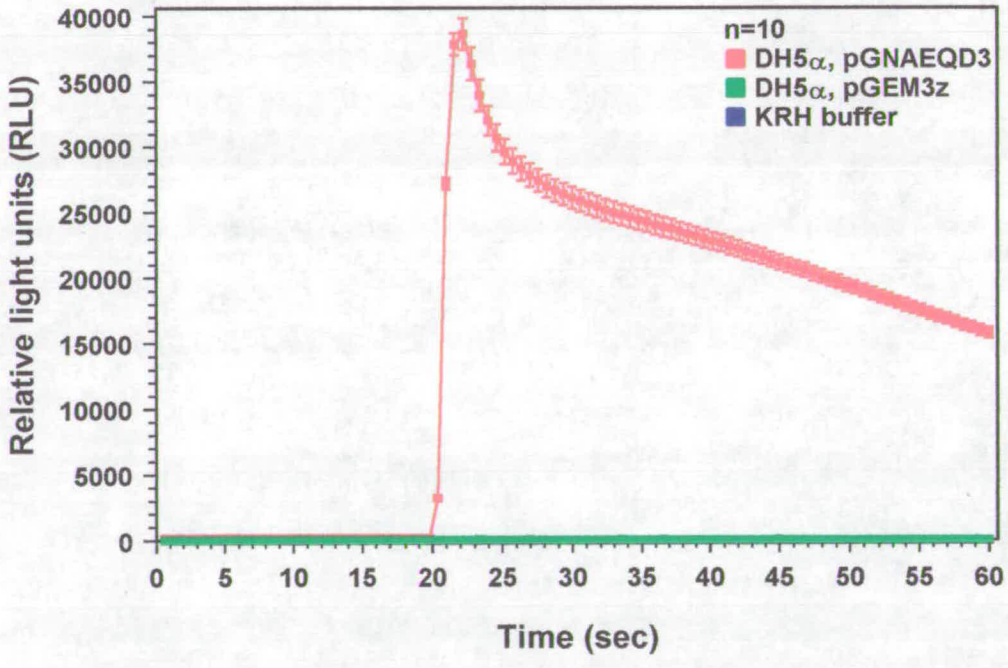
From sequencing analysis, it seemed apparent that pGNAEQD3 contained the correctly synthesised gene. As a means of verifying this, expression of *gnaeqD* in *E. coli* was used to prove that the gene product was functional prior to producing fungal expression vectors.

A simple method of determining whether apoaequorin was functional from *gnaeqD* was to use the cloning vector in which it was produced as an expression vector. Due to the whole gene being inserted anti-clockwise, it was possible that apoaequorin would be expressed from the *lacZ* promoter. However, a negative result would not necessarily mean that *gnaeqD* did not produce active aequorin as the vector was not designed for expression of an inserted gene, and the cloning method used did not specifically place the *gnaeqD* in frame with the 3' portion of *lacZ*. As a simple, quick assay, this was attempted.

5 ml cultures of GNAEQD3 and DH5α transformed with the empty cloning vector, pGEM3z, were grown overnight in LB with ampicillin, and expression from the *lacZ* promoter induced by the addition of IPTG to a final concentration of 0.5 mM, and the cultures incubated for a further 3 h. Cells were then harvested by centrifugation

for 5 min in a microcentrifuge, and resuspended in half the volume with Krebs-Ringer Hepes buffer (KRH; 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM Hepes, pH 7.5; Knight *et al.*, 1991). 100 µl aliquots of cells were then added to the wells of a multiwell plate and coelenterazine added to a final concentration of 2.5 µM. The plate was incubated at 37 °C for 4 h prior to luminometry of the samples. To elicit a response from any active aequorin, the wells were injected with 100 mM $CaCl_2$ with 1% Nonidet P40 (Boehringer Mannheim), a detergent that permeabilises the plasma membrane (Knight *et al.*, 1991). The results are shown in Figure 3.14.

Figure 3.14: Expression of aequorin in *E. coli*. Both controls (cell resuspension/aequorin reconstitution buffer (KRH) and pGEM3z-transformed DH5α have light levels that are effectively zero on the axis shown. Injection of 100 mM $CaCl_2$/ 1% Nonidet P40 occurs at 20 s in all samples. Error bars are SD(n-1).



From the luminometry studies, it appears that active aequorin is formed in the clone containing pGNAEQD3. With the addition of a detergent and external Ca^{2+}, a clear

response is seen in cells containing the apoaequorin gene which is absent from the two controls. The comparatively slow rise in the light response (2-3 sec before the light response reaches its apex) can be attributed to the effect of NP40 slowly permeabilising the cell membranes and the concomitant increase in intracellular Ca^{2+}.

3.5 Discussion

This chapter describes the design of a synthetic apoaequorin gene with optimal codon bias for *A. niger* and the successful production of the synthetic gene. Preliminary results have shown that the synthetic gene, *gnaeqD*, produces functional aequorin in *E. coli*.

There were two major problems that had to be overcome in this work. The presence of a large number of mutations in the synthetic gene halves, *gnaeqDA* and *gnaeqDB* meant that a lot of clones had to be sequenced, and an extra step had to be inserted into the planned method of synthesis to produce two correct gene halves. If time had not been a constraint, then the mutations could have investigated further by altering the PCR conditions and comparing sequenced clones from each PCR for the amount of mutations. However, it is quite likely that the mutations originated from errors in the synthetic oligonucleotides rather than from PCR. Oligos of 100 bp, as used, are close to the maximum size that oligos can be produced, and due to their size, they have a large chance of at least one base being missed out or incorrect. Most of the errors found were single base mutations or deletions (Table 3.10), which would fit this theory. An alternative method of producing the synthetic gene would probably have overcome the number of mutations. This would have involved designing shorter oligos that almost completely overlapped along the gene sequence. PCR of these oligos would have, in theory, produced far fewer mutations due to the oligos being shorter and the primers covering most of the sequence. However, this would

have meant synthesising far more base pairs of synthetic oligonucleotides, which would have been expensive.

Another problem was encountered when attempting to verify the sequence of the clones. Due to the high GC content of the synthetic gene, manual sequencing was difficult. Compressions occurred at specific sites within the sequence, and two or three of these sites appeared to exist in both gene halves. Resolving the actual sequence at such sites was difficult. The use of the guanidine analogue, inosine, was fairly successful. However, for final verification of clones, automated sequencing was used. This almost always resolved compressions. Interestingly, the manual sequencing of the whole gene constructs was easier than that of the half gene constructs.

Since this work was planned and carried out, the same problem has been tackled with the Green Fluorescent Protein (GFP) which is from the same species as aequorin, for expression in the fungi *Candida albicans* and *Saccharomyces cerevisiae* (Cormack *et al.*, 1997). The results obtained with GFP in *C. albicans* showed no expression with the native gene but expression with the synthetic, yeast-enhanced GFP (yEGFP). The results obtained from the codon optimisation of GFP are very similar to those presented in this thesis.

4. COMPARISON OF APOAEQUORIN EXPRESSION LEVELS IN *NEUROSPORA*

4.1 Introduction

Previous attempts to produce a high level of apoaequorin expression in *N. crassa* have been largely unsuccessful (Collis, 1996). Expression of apoaequorin A (*aeqA*) was attempted under two different *N. crassa* promoters, the glucose repressible gene-1 (*grg*-1) promoter and a truncated malate synthase (*ms*) promoter. The *ms* promoter (P*ms*) used was an 800 bp fragment of the whole P*ms* (Sheffield, 1994). The truncated P*ms* does not possess *cre*A (carbon catabolic repression) or *amd*19 (acetate induction) consensus sequences (Mizote *et al.*, 1996), and consequently it is a constitutive promoter. The *aeqA* expression vector produced with this promoter contained two AUG start codons, one from P*ms*, the other belonging to *aeqA*. Sequence analysis showed they were both in-frame with a 7 codon linker separating them. Subsequent investigation suggested that the presence of two start codons made little difference to apoaequorin expression (Collis, 1996). Incorporation of the *ms* promoter (P*ms*) expression cassette was selected for using the *E. coli* plasmid gene encoding hygromycin B phosphotransferase which confers resistance to hygromycin B, an antibiotic produced by *Streptomyces hygroscopicus*.

The *grg*-1 promoter (P*grg*-1) (McNally and Free, 1988; Wang *et al.*, 1994) is a 1.4 kb sequence which shows strong induction in glucose deprived conditions. Under de-repressed conditions, as during growth on sucrose, P*grg*-1 has been shown to direct calf preprochymosin gene expression in *N. crassa* (Nakano *et al.*, 1993). Expression using this promoter is controlled by the gene's own start codon. The *grg*-1 vector contained a phosphoinothricin acetyltransferase gene (*bar*) from *S. hygroscopicus* which conferred resistance to the glutamine synthetase inhibitor, phosphoinothricin.

Both transformation selection procedures were successful, yet expression from both promoters was found to be far less than previously observed in *N. crassa* with other heterologous genes (Nakano *et al.*, 1993; Sheffield, 1994). The observation that *aeqA* mRNA was present in transformants together with studies on apoaequorin degradation suggested that codon bias may be the cause of the low expression levels in *N. crassa* (Collis, 1996).

To test the hypothesis of codon bias, expression of the synthetic gene, *gnaeqD*, was compared to the expression of *aeqA* in *N. crassa*. One of the expression vectors previously used for *aeqA* expression was chosen (pBARGRG-1) for construction of the synthetic gene expression system, and the same transformant selection used (*bar*). The fact that previous work was carried out with the native *aeqA* gene and that the synthetic gene was based upon *aeqD* was expected to make little difference, due to the large similarities between the two isoforms (see Section 3.2.2).

For analysis of fungal transformants, a source of apoaequorin protein was required. This was needed to quantify the production levels of protein in the fungal transformants by comparison of luminescence levels in fungal protein extracts with the luminescence of known concentrations of pure aequorin. Also, purified apoaequorin was required to raise antibodies for Western analysis of fungal transformants (see Section 5.4.1). Pure apoaequorin is available commercially, and would have been suitable for *in vitro* quantification. For antibody production, a large amount of pure protein is required and this would have been expensive to purchase. Therefore, a suitable method of producing and purifying apoaequorin was required. *E. coli* was chosen as a host organism for overexpression, and the method chosen for purification was to produce apoaequorin as a His-tagged protein. Adding several histidine residues to the carboxy (C-) or amino (N-) terminus of a protein allows for rapid and simple purification of the protein using the affinity of Nickel (Ni^{2+}) to bind histidine. By using an agarose-Ni^{2+} complex to produce a solid substrate containing free Ni^{2+} ions, a column can be created through which a whole cell extract can be

passed. Proteins containing histidine residues will bind to the free Ni^{2+} ions, and therefore be trapped in the column. These proteins can then be eluted from the column in order of their affinity to the Ni^{2+} (i.e. the more His residues, the greater the affinity) using either a pH gradient or an imidazole gradient, which competes with the His residues for the Ni^{2+} ions. The drawback to this method is that the purified protein is modified, with either a C- or N- terminal addition of approximately 20 amino acids. This may affect the activity of the mature protein, which is essential for protein quantification assays. Addition of a specific protease site in the linker sequence allows removal of almost all of the extra amino acids if they affect the activity of the protein. The C-terminal of aequorin has previously been suggested to be important for activity (Watkins and Campbell, 1993).

The aims of the work described in this chapter were to:

1. Produce an identical expression vector to that used with the native apoaequorin gene using the synthetic gene, and transform *N. crassa* with the synthetic expression vector.

2. Compare expression of synthetic apoaequorin with that of native aequorin in the best transformants with each expression vector.

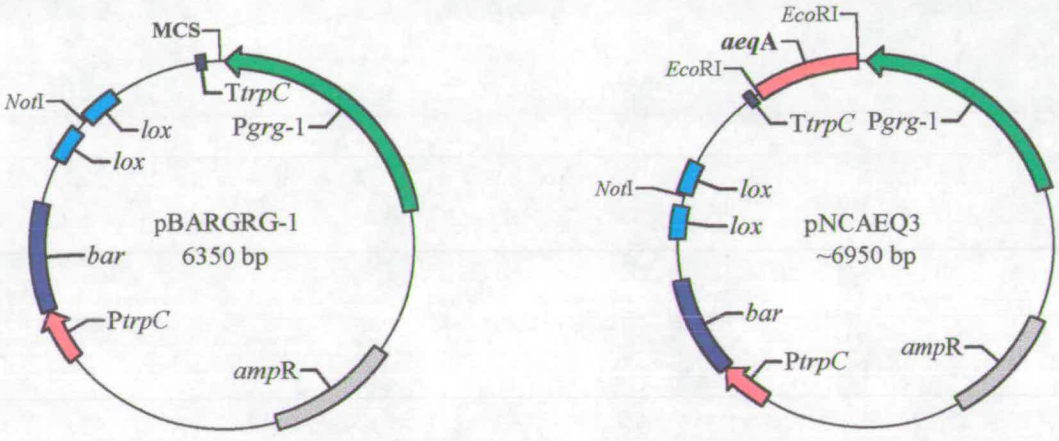
3. Produce His-tagged apoaequorin in *E. coli* to purify apoaequorin for quantifying *in vitro* luminescence and raising antibodies (Section 5.4.1).

4.2 Production of *Neurospora* expression vector

The *Neurospora* expression vector pBARGRG-1 containing the native *aeqA* gene, called pNCAEQ3 (Collis, 1996) was used to construct the synthetic gene expression vector. An outline of the original vector and pNCAEQ3 is given in Figure 4.1.

AeqA could be excised from pNCAEQ3 using *Eco*RI, which could also be used to excise *gnaeqD* from pGNAEQD3 (Section 3.3.4) allowing simple cloning of the synthetic gene into the empty pBARGRG-1 vector.

Figure 4.1: *Neurospora* expression vector pBARGRG-1 and the aequorin expression vector, pNCAEQ3



Key: MCS; multiple cloning site containing *Eco*RI site, T*trpC*; terminator region of *trpC* gene of *A. nidulans*, P*grg*-1; truncated promoter of *grg*-1, *amp*R, ampicillin resistance gene, P*trpC*; promoter region of *trpC* gene, *bar*; basta-ignite resistance gene, *lox*; *lox-Not*I-*lox* site-specific recombination sequence for excision of plasmids from λ/plasmid hybrids (Brunelli and Pall, 1993).

gnaeqD was excised from pGNAEQD3 (Figure 3.13) and both this approximately 630 bp fragment and the *Eco*RI-linearised, phosphatased vector, pBARGRG-1 (with *aeqA* excised) were gel purified and the concentration of the two purified fragments estimated by agarose gel electrophoresis. Ligation reactions were then prepared and the mixtures used to transform DH5α cells which were plated on LB plates containing ampicillin. Twenty successful transformants were picked and miniprepped. Restriction digest analysis using *Eco*RI confirmed whether there was a correct sized insert present, and further analysis using *Bam*HI determined the orientation of the insert. *Bam*HI cuts at the 5' end of *gnaeqD* and at the 5' end of P*grg*-1, so correct orientation would yield a fragment of approximately 600 bp. A successful transformant containing *gnaeqD* in the correct orientation was found and

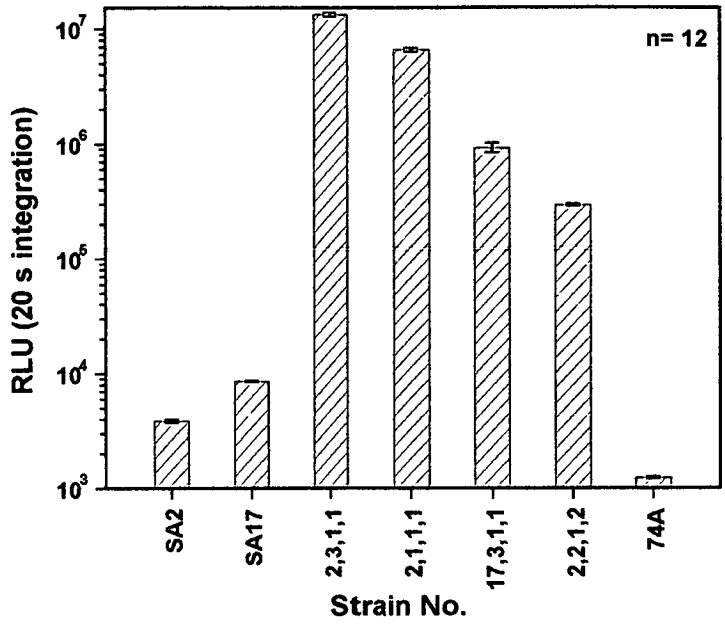
called pNCAEQ4, and a plasmid maxiprep made to bulk up enough DNA for transformation of *N. crassa*.

4.3 Transformation and screening

N. crassa strain 74A protoplasts were transformed by electroporation using 2 μg pNCAEQ4 per 6 x 10^6 protoplasts, and the transformation mixture plated out on osmotically protected *bar*$^+$ selection plates (Section 2.11). Non-abortive transformants were found to produce quite large colonies, and these were isolated and transferred to non-selective slants. As an initial screen, the primary transformants were inoculated into 50 ml VS and the flasks incubated at 25 °C in the dark for 4 days. Mycelia were harvested and whole cell protein extracts prepared (Section 2.12.1.2). Protein concentration was determined (Section 2.12.2) and *in vitro* luminometry assays carried out on 10 μg protein samples (section 2.16.4). Transformants which showed the largest response were then chosen to be purified. Since each protoplast in the transformation mix can be expected to be multi-nucleate and transformation can only be expected to introduce DNA into one nucleus, serial isolation of conidia was performed to purify the transformants (Section 2.11.3).

Purified transformants were then screened by luminometry of whole cell protein extracts. A sample of data for two high expression primary transformants and purified transformants is shown in Figure 4.2.

Figure 4.2: Luminometry of whole cell soluble protein extracts from *N. crassa* transformants



Key: Samples prepared as described in text. Data given as a 20 s integration of Relative Light Units (RLU) after addition of $CaCl_2$ to a final concentration of 50 mM. Strain 74A is the untransformed control. Strains SA2 and SA17 are the two high expression primary transformants from which the high expression purified strains are derived. Purified strains are represented by their serial isolation numbers. Error bars are SD(n-1).

In total 32 primary transformants were analysed. Of these, 2 transformants were purified. Purification increased the levels of light compared to the primary transformants (Figure 4.2), probably by removing untransformed nuclei and therefore increasing the total number of nuclei per weight of mycelia that are capable of producing *gnaeqD* mRNA. Two transformants (2,3,1,1 and 17,3,1,1) which gave the highest luminescence levels from two primary transformants (SA2 and SA17, Figure 4.2) were chosen for comparison with native aequorin expression in *N. crassa*.

4.4 Comparison of expression levels between native and synthetic apoaequorin

Fresh conidia from *N. crassa* transformants containing the synthetic apoaequorin gene (transformants 2,3,1,1 and 17,3,1,1) and the high expression native apoaequorin

containing 50 ml VS, which were incubated at 25 °C for 4 days. Mycelia were harvested from each flask, whole cell protein extracts prepared and active aequorin formed by incubation with coelenterazine. Luminescence was monitored for 20 seconds after stimulation with 100 mM $CaCl_2$. The results are displayed in Table 4.1.

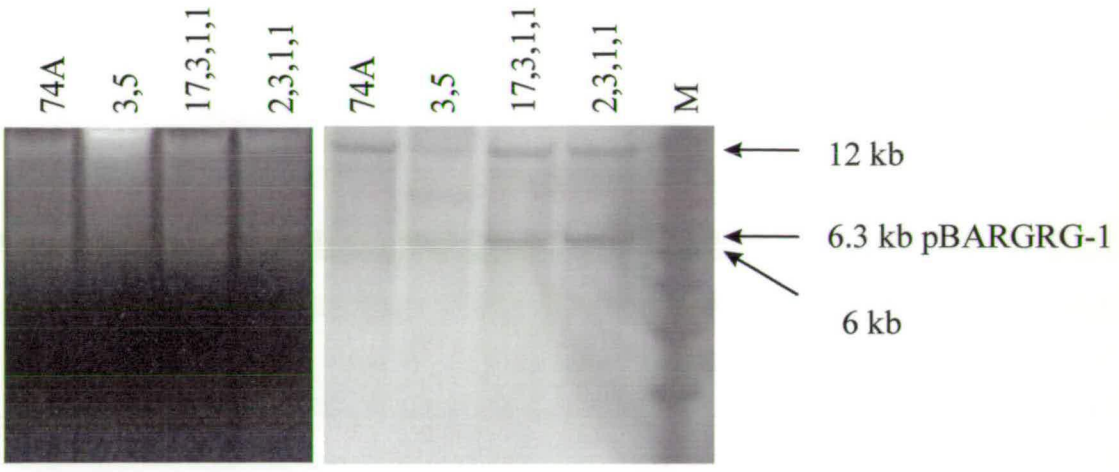
Table 4.1: *in vitro* comparison of synthetic and native aequorin expression

Transformant strain	RLU (20 s integration) mean ± SD(n-1)
untransformed control	
74A	291.5 ± 41.3
synthetic aequorin	
2,3,1,1	790,712.1 ± 71,086.1
17,3,1,1	50,664.6 ± 14,743.6
native aequorin	
3,5	2,833.5 ± 522.5

Key: n = 8, *p* value between strains = 5.36×10^{-28} (Single factor ANOVA).

The *p* value (Table 4.1) obtained for the data shows that the differences between the strains are very highly significant. It can clearly be seen that the expression levels appear higher in the transformants possessing the synthetic gene, which could be attributable to the codon bias. However, the copy number and integration site of the inserted expression cassettes could also affect the expression levels. To compare copy number in the best native and synthetic transformants, Southern blots were prepared of *Eco*RI digested genomic DNA. For each transformant, 2 μg of *Eco*RI digested genomic DNA was loaded onto a 0.8% agarose gel which was electrophoresed until the smallest fragments reached the bottom of the gel. A blot was prepared, and this was then hybridised overnight at 50 °C with a ^{32}P-labelled probe using the *Eco*RI-digested vector backbone as the probe. Washes and detection were then carried out as described in Chapter 2. The results are shown in Figure 4.3.

Figure 4.3: Southern analysis of *N. crassa* transformants hybridised with ^{32}P-labelled pBARGRG-1



Key: marker is 1 kb DNA ladder (Gibco, UK). 74A is the untransformed control strain. 3,5 is the native aequorin transformant. 17,3,1,1 and 2,3,1,1 are the synthetic aequorin transformants. Marker positions of 6 kb and 12 kb are indicated (marker is 1 kb DNA ladder).

*Eco*RI excises *gnaeqD* from pBARGRG-1, and therefore the probe used is approximately 6.3 kb. The transformants show a band of this size which is absent from the control strain, 74A. There is also a larger band of approximately 12 kb. It is possible that this could be inverted repeats of pNCAEQ34 integrated into the genome. However, this band is also present in the control strain. To test whether the control strain was contaminated, protein extracts were prepared from some of the mycelia harvested from the flasks that were used for the genomic DNA preps. Luminometry analysis of the protein extracts was carried out as previously and only background levels of light were obtained from 74A (data not shown). Therefore it was concluded that the 12 kb band was background binding. This is probably due to using a large sized probe. A large part of the vector backbone is of pUC origin (Pall and Brunelli, 1994), and therefore it is not surprising that there is binding to the marker DNA, which is built from repeats of plasmid DNA (Gibco, UK).

It can be seen that the number of copies of the expression cassette are approximately the same between each of the transformant strains. Therefore this cannot be expected to have had an influence on the expression level disparity observed in the

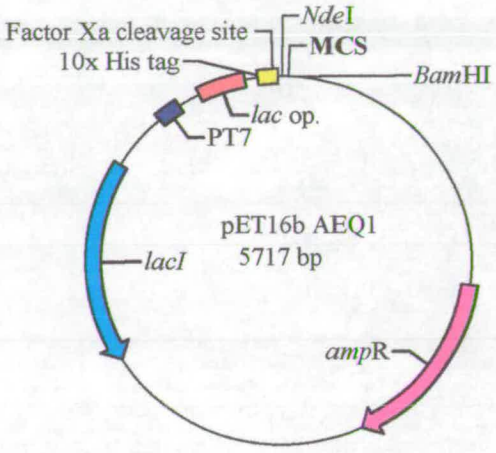
luminescence studies (Figure 4.2). The native gene transformant, 3,5, shows less binding of the pBARGRG-1 probe, but as can be seen from the EtBr-stained gel, there has not been complete digestion of the DNA in 3,5. It can be concluded that there is an approximately equal copy number in both the synthetic gene transformants and in the native gene transformant. The reason for this is unknown, as a large number of primary transformants were analysed for both transformant types (30 transformants for pNCAEQ3 (Collis, 1996), 32 for pNCAEQ4), and it would be expected that high expression strains obtained with both vectors would contain approximately equal, high copy numbers. The same transformation procedure was used for both vectors and the same method was followed for transformant selection and screening.

4.5 Quantification of aequorin luminescence

Purified apoaequorin was produced in *E. coli* by creating a His-tagged fusion protein which could then be overexpressed and purified using a Ni^{2+}-NTA column (Qiagen, UK). Several vectors are available which direct overexpression of a protein fused to either a C- or N- terminal His tag in *E. coli*. One such vector is pET16b (Figure 4.4, Novagen, UK), which directs expression of the fusion protein using the T7 RNA polymerase promoter. Use of a suitable expression host, such as BL21 (DE3) which contains the T7 RNA polymerase gene in the chromosome, allows for expression using the T7 promoter. Expression of T7 RNA polymerase is controlled by the *lacUV5* promoter, which is inducible by the addition of IPTG (12 mg/ 100 ml) to the media. The vector also contains *lac*I, which encodes the *lac* repressor. The LacI protein binds to the *lac* operator which is situated downstream of the T7 promoter in pET16b (Figure 4.4), preventing transcription. BL21 (DE3) also lacks the regions encoding the *ompT* outer membrane protease and the *lon* protease, which help contribute to the stability of the expressed protein.

encoding the *ompT* outer membrane protease and the *lon* protease, which help contribute to the stability of the expressed protein.

Figure 4.4: pET16b, His-tag expression vector



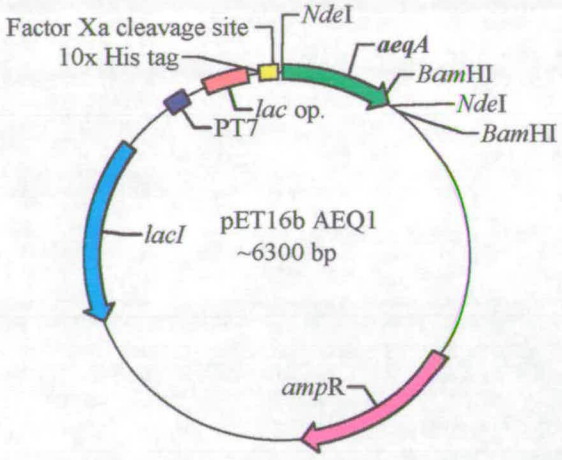
Key: *Nde*I and *Bam*HI restriction sites in the multiple cloning site (MCS) shown. *amp*R; ampicillin resistance gene. *lacI*; *lac* repressor gene. PT7; T7 RNA polymerase promoter. *lac* op; *lac* operator. 10x His tag; DNA encoding 10 Histidine residues in-frame from start codon. Factor Xa cleavage site; protease cleavage site for removal of His tag.

4.5.1 Production of His-tagged apoaequorin

Due to the sequences of pGNAEQD and pET16b, it would be very difficult to insert *gnaeqD* in-frame in pET16b. Both vectors use incompatible restriction enzymes around the AUG codon (pET16b has an *Nde*I site[5'CA^TATG3'] and pGNAEQD has a *Bsp*HI site [5'T^CATGA3']). PCR could have been used to alter the restriction site at the start codon. However, an alternative method was to use the native *aeqA* gene, which has already been altered to include an *Nde*I site over the start codon and 3' of the gene sequence (Collis, 1996). As outlined in Chapter 3, there are 19 amino acid differences between AEQA and AEQD, but since both proteins have identical activity it would not matter which was used for quantification of aequorin levels. The differences could possibly affect the affinity of an anti-aequorin antibody raised with one isoform. However, it was planned to use the antibody to detect both isoforms, and therefore there could be a problem whichever isoform was used for raising antibody.

Modified native *aeqA*, containing *Nde*I sites at the start codon and at the 3' end of the gene was present in pbsAEQ, a bluescript-based cloning vector (Collis, 1996). Using *Nde*I, *aeqA* was excised and the fragments gel-purified. The His tag expression vector pET16b was linearised with *Nde*I, phosphatased and then gel purified to remove salts and modifying enzymes. Ligations were performed using ratios of 3:1 and 5:1 insert:vector with suitable controls also set up. 10 µl of each ligation mix was then used to transform chemically competent DH5α cells, and a selection of positive transformants picked. These were miniprepped, and correctly orientated inserts determined by restriction analysis with *Bam*HI. This cuts 3' of the vector ligation site and near the 3' end of *aeqA*, and therefore correct orientation of the insert will excise an approx. 100 bp fragment. Incorrect orientation would release an approx. 500 bp fragment and religated vector would only be linearised. A correctly ligated construct, named pET16b-AEQ1, was found. A representation of pET16b with the native *aeqA* fragment inserted is shown in Figure 4.5.

Figure 4.5: pET16b with *aeqA* correctly inserted at *Nde*I site in the multiple cloning site.

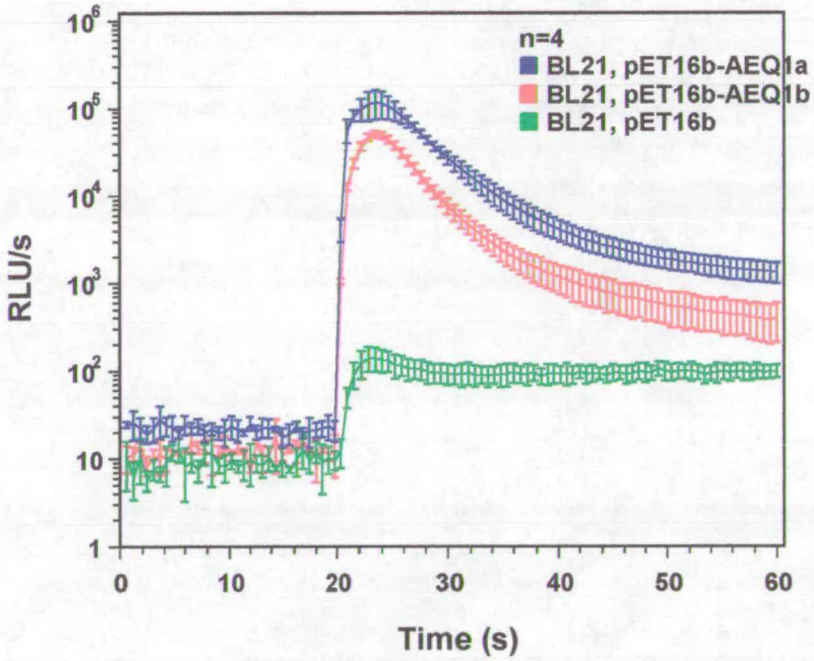


Key: As described for Figure 4.4, with all *Nde*I and *Bam*HI sites shown. *aeqA*; native apoaequorin A gene inserted in the correct orientation at the *Nde*I site of the MCS.

The successful clone was transformed into chemically competent BL21 (DE3) cells. Two positive clones, pET16b-AEQ1a and pET16b-AEQ1b, were picked and tested *in vivo* to determine whether active apoaequorin was produced from pET16b-AEQ1 prior to protein purification. This involved growing a miniculture overnight, then

using this to subculture 1:10 to a fresh miniculture. This was incubated for 2 hours then IPTG (0.6 mg per 5 ml) and coelenterazine (final conc. 2.5 μM) added. The culture was incubated for a further 3 hours at 37 °C. Cells were harvested by centrifugation and resuspended in one tenth their original volume with KRH buffer (Section 3.4). 100 μl of cell suspension were then placed in wells of a multiwell plate and aequorin activity assayed by luminometry. Each well was stimulated by the addition of 100 mM $CaCl_2$, 1% Nonidet P40 (NP40) at 20 s. The results show that both picked clones express active aequorin (Figure 4.6). The difference between the two clones may be due to different cell concentrations as the data is only qualitative. pET16b-AEQ1a was chosen for apoaequorin purification.

Figure 4.6: *in vivo* luminescence of active aequorin in *E. coli* BL21 cells from pET16b vector



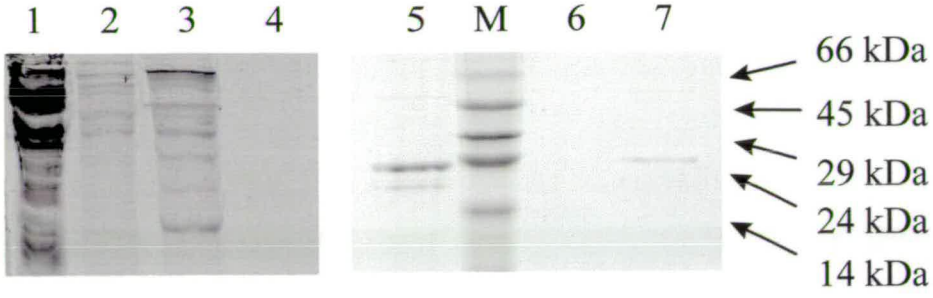
4.5.2 Purification of His-tagged apoaequorin

Soluble cell extract was prepared from large volume cultures of BL21 pET16b-AEQ1a as described in Section 2.12.1.1. The protein suspension was placed onto Ni^{2+}-NTA resin columns and purified using an imidazole gradient as described in Section 2.12.5. The eluates obtained with 180 mM imidazole, which were expected to contain purified His-tagged apoaequorin, were then concentrated by TCA precipitation, and samples from eluates analysed by SDS-PAGE using a 12.5% polyacrylamide gel (Figure 4.7).

As can be seen in Figure 4.7, the 120 mM imidazole washes were adequate to remove contaminating proteins, as there is no visible protein present in the second 120 mM imidazole wash (lane 4). Also, after elution of the final bands with 180 mM imidazole (lane 5), no further protein can be seen with the 240 mM imidazole wash (lane 6).

TCA precipitation of the pooled 180 mM imidazole eluates from 8 litres of culture gave a protein concentration of approx. 0.3 mg/ml. For antibody production, a final concentration of 1 mg/ml was required, and therefore to further concentrate the sample Vivaspin columns were used as described in Section 2.12.6.2. Sample purity was estimated by SDS-PAGE (Figure 4.7, lane 7), and the final concentration adjusted to 1 mg/ml. Gel analysis revealed a doublet band had been purified using the Ni^{2+}-NTA columns (lane 5), although this was not seen after concentration in Vivaspin columns (lane 7). The purified band has a molecular weight of almost 24 kDa, which is the size expected of apoaequorin with a His tag and linker. Luminometry assay of the purified protein after incubation with coelenterazine proved that the protein obtained was active aequorin (Figure 4.8).

Figure 4.7: Coomassie-stained polyacrylamide gels of His-tag apoaequorin purification



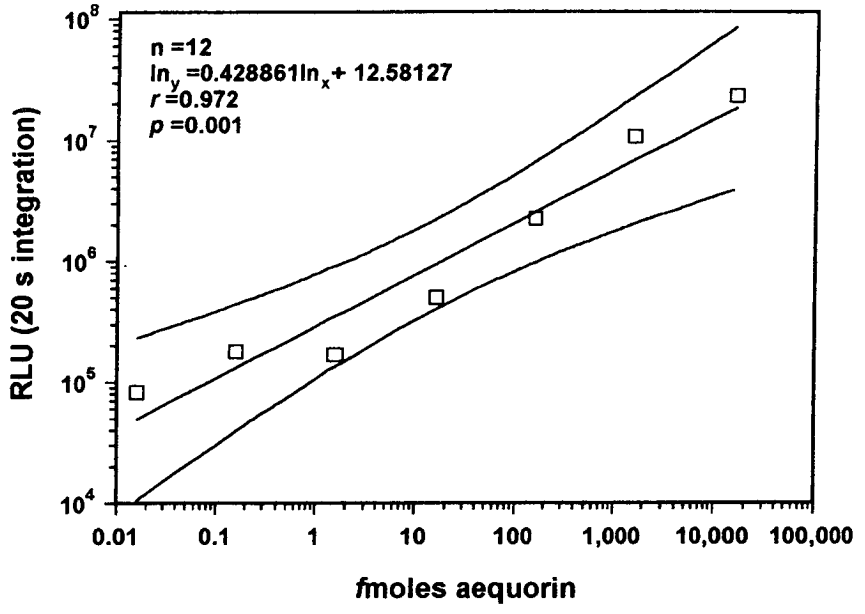
Key: Lanes loaded in the following order: 1, loaded sample, 2; 0 mM imidazole wash; 3, first 20 mM wash; 4, second 120 mM wash; 5, TCA-precipitated 180 mM eluate sample (5 µl loaded); M, marker (sizes shown in figure); lane 6, TCA-precipitated 240 mM eluate sample (5 µl loaded); 7, concentrated eluate from Vivaspin column (1 µl loaded). All references to molarity refer to imidazole concentration. Lanes 1-4 loaded with 10 µl sample. TCA-precipitated samples are concentrated 5 times (therefore a 5 µl load is equivalent to 25 µl).

The purified protein was dialysed in 3 changes of phosphate-buffered saline (PBS) and the concentration determined.

4.5.3 Standard curve and quantification of aequorin luminescence

Purified His-tagged apoaequorin was used to quantify the amount of aequorin luminescence obtained from transformed cells analysed in the luminometer used in this study. A dilution series, from 16 *p*moles to 0.016 *f*moles of aequorin was made for the protein sample, and each dilution incubated with 2.5 µM coelenterazine in KRH buffer at 25 °C for four hours prior to stimulation. Wells were injected with 100 mM $CaCl_2$ and luminescence monitored. The luminescence obtained over 20 s after stimulation was integrated and the results compared to the known concentration of protein (Figure 4.8). The r value obtained shows that the data is significant at $p = 0.001$, which shows that the data fit a straight-line relationship with 99.9% confidence.

Figure 4.8: Standard curve of aequorin light emission



Key: Line of best fit shown with 99.9% confidence band limits.

Using the standard curve obtained from the purified protein, the total amount of aequorin present in soluble whole cell extracts was determined from the luminescence obtained from the samples (Table 4.1). The calculated levels of aequorin are shown in Table 4.2.

Table 4.2: Quantification of aequorin from soluble whole cell extracts of *N. crassa*

Transformant strain	RLU (20 s integration after stimuation)	*f*moles aequorin per 10 µg protein extract	*f*g aequorin/ µg protein
synthetic aequorin			
2,3,1,1	790,712.1	10.282	22,620.6
17,3,1,1	50,664.6	0.017	37.32
native aequorin			
3,5	2,833.5	2.04×10^{-5}	0.045

Key: 1 *f*mole aequorin weighs 2.2×10^{-11}g. Luminescence data from Table 4.1.

4.6 Discussion

Expression levels of native and synthetic apoaequorin have been compared in *N. crassa*. The synthetic gene appears to produce a significantly higher level of apoaequorin expression than the native gene, with the figures showing approx. 5 x 10^5 increase in aequorin concentration between the two best transformants (strain 2,3,1,1 for the synthetic gene and strain 3,5 for the native gene, Table 4.2). The gene copy number present in the two strains appears to be aproximately equal (Figure 4.3). In comparison to apoaequorin production in other species, native AEQA is produced to a concentration of 4,740 *fg*/μg total soluble protein in *N. plumbaginifolia* (Collis, 1996), and the best synthetic gene transformant produced AEQD approx. 5 times that concentration (strain 2,3,1,1, Table 4.2).

There are a number of factors which have not been analysed in this brief investigation of apoaequorin expression levels. Firstly, validation that synthetic apoaequorin was definitely ligated into the expression vector by DNA sequencing was not carried out. The expression levels obtained between transformants was always seen to be varied. Therefore it could be argued that the number of transformants analysed was not enough to find high expression transformants containing the native expression cassette. This cannot realistically be seen to be the case. Thirty primary transformants were analysed for the native gene expression (Collis, 1996) and 32 primary transformants analysed for the synthetic gene transformation. The chances of the synthetic gene transformants being true high expression transformants and none of the native gene transformants truly being high expression transformants is therefore very slim. In addition, the frequency at which high expression transformants were obtained was seen to be approximately the same for both the synthetic gene vector and the native gene vector transformations. In this study and in the native gene transformations (A. Collis, pers. comm.), it was found that approx. 1 in 20 of picked primary transformants were high expression strains. Why this frequency is seen is unknown, but can be expected to be due to the combined effect of the copy number and the integration site. A simplified

explanation could be that random integration only places the expression cassette(s) in an area of the genome from which high expression is possible one in twenty times. The copy number in each transformant was not analysed in great depth in this study. However, Southern analysis did show a greater copy number in the best synthetic gene transformants compared to the best native apoaequorin transformant (Figure 4.3). Phosphoimaging could be used to obtain an accurate estimate of actual copy number in the different strains. Previous studies have shown that copy number is not always directly proportional to expression level due to the random integration site (Baron *et al.*, 1992; Finkelstein *et al.*, 1989; Verdoes *et al.*, 1993).

Overall it appears that the bias of codons in the heterologous gene does make a difference to the expression level of the protein. A means of determining this further would have been to isolate phosphoinothricin resistant spores from sexual crossing of the transformants. By meiosis, the tandemly integrated copies of the expression cassettes would most likely have been recombined out of the genome to leave a single integrated copy. This would have allowed the removal of gene copy number from the uncertainty surrounding the effect of codon bias. The site of integration is known to be more important for expression however, and this matter would not be resolved by sexual crossing.

The findings of Cormack *et al.*, (1997) showed that producing a synthetic GFP gene with preferential codon usage for *C. albicans* overcame the lack of expression in *C. albicans*, and also produced greater levels of expression in *S. cerevisiae*. This was shown to not be due to the non-canonical serine codon found in *C. albicans*. This has strong parallels with the work presented in this chapter; GFP and apoaequorin are both genes from *A. victoria* and both have been expressed to a greater extent using the codon preference of the respective fungi. The results published by Cormack *et al.* for GFP expression corroborate the findings described here for apoaequorin.

Also described in this chapter is the purification of His-tagged apoaequorin from *E. coli*. The yields obtained were quite low for protein overexpression in *E. coli*, although insoluble cell extract was not examined for apoaequorin, which may have precipitated out as poly-inclusion bodies. The purified protein was shown to be active after incubation with coelenterazine, and allowed a standard curve of aequorin luminescence to be determined.

5. PRODUCTION OF TRANSGENIC *ASPERGILLUS* EXPRESSING SYNTHETIC APOAEQUORIN

5.1 Introduction

Fungi of the genus *Aspergillus* are used in industrial fermentation for production of homologous and heterologous enzymes (Finkelstein, 1991; May, 1992) and therefore gene expression studies have been carried out to try and improve protein production (van den Hondel *et al.*, 1991). Transformation procedures have been developed and optimised to enable strict selection for a high gene copy number in stable transformants (van Gorcom *et al.*, 1994). *Aspergilli* have been successfully used to produce high levels of proteins from other fungi and also from higher eukaryotes such as human, bovine and chicken (see Table 1.3). Heterologous protein production levels are often far lower than those that can be obtained for homologous proteins. The explanations for this are often never fully determined, although the limiting steps have been proposed for several proteins. Usually, transcription is not seen as a major limiting step as the use of strong fungal promoters for heterologous expression allows for a high level of mRNA production. Translation initiation, elongation and protein folding are all steps which have been suggested as limiting factors in heterologous protein production. It has been postulated that altering a heterologous genes sequence to mimic that of a homologous gene could alleviate the limitations upon translation initiation and elongation (Collis, 1996). This has been done as described in Chapter 3. Altering the protein sequence would be far more complicated, as the effects upon the proteins activity would be unknown, and quite probably deleterious. Therefore, in the synthetic apoaequorin gene this has been left unaltered.

The synthetic gene has been cloned into a constitutive expression vector for cytosolic expression in *Aspergilli.* This was done using a pre-constructed vector, pAN52-1, from TNO, The Netherlands. It contains the glucose-6-phosphate dehydrogenase promoter (P*gpdA*) from *A. nidulans* (Kolar *et al.*, 1988) attached to a small multiple cloning site, which is fused 3' to the terminator region of the trifunctional tryptophan biosynthesis enzyme, *trp*C from *A. nidulans* (Mullaney *et al.*, 1985). The selectable marker ampicillin is present which allowed the vector to be cloned in *E. coli.* For selection in *Aspergilli*, a suitable selection marker was ligated into the plasmid. Co-transformation was carried out with a second selectable plasmid to increase the stringency of selection for positive transformants. Selection and purification of transformants is described, and the subsequent isolation of high transgene expression strains is outlined.

5.2 Expression vector production

pAN52-1 was a suitable expression vector with appropriate restriction sites for insertion of *gnaeqD* (Figure 5.1). A modified version of pAN52-1 was used for creating the apoaequorin expression vector. This was pNOM102 (Figure 5.2), which contained the *E.coli* β-glucuronidase gene, *uidA*, in the cloning site (Punt et al., 1991). Restriction digestion with the appropriate restriction enzymes for inserting *gnaeqD* would excise *uidA*, leaving the pAN52-1 backbone.

Figure 5.1: *gnaeqD*, showing position of restriction sites used for cloning

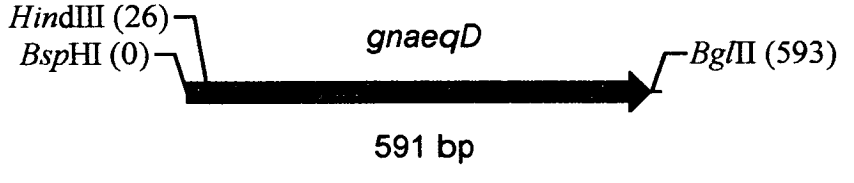
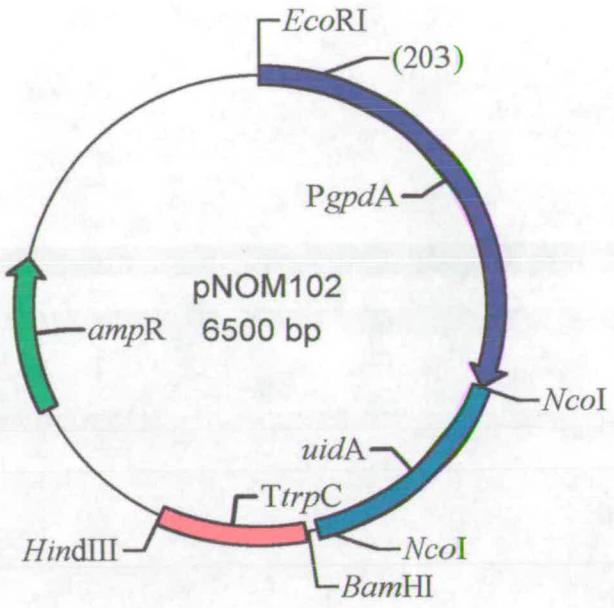


Figure 5.2: pNOM102, showing selected restriction sites



Key: P*gpd*A; glucose-6-phosphate dehydrogenase promoter. T*trp*C; terminator region of *trp*C. *amp*R; ampicillin resistance gene. *uid*A; β-glucuronidase gene of *E.coli*.

The enzymes used for excision of *uidA* from pNOM102 were *Nco*I and *Bam*HI. Removal of *gnaeqD* (Figure 5.1) from pGNAEQD3 used *Bsp*HI and *Bgl*II. *Bsp*HI and *Nco*I leave compatible overhangs at the cleavage sites which allows religation of fragments cut with the two enzymes. The same is true for *Bam*HI and *Bgl*II. The correct DNA fragments were isolated by agarose gel purification using Gene Clean II, and ligations performed between *gnaeqD* and the linearised, phosphatased pNOM102 vector to produce the vector pAEQ1. Theoretically, the ligation inserted *gnaeqD* in the correct orientation under the control of its own start codon. Competent DH5α cells were transformed by electroporation using the ligation mixtures. Miniprepped transformants were screened by restriction analysis using *Hin*dIII, which would excise the *gnaeqD* insert and T*trp*C as a single fragment if *gnaeqD* was present, and would otherwise linearise the empty plasmid. Three successful clones, pAEQ1-1, pAEQ1-2 and pAEQ1-3 were chosen for further work.

Prior to production of the final vector for transformation, the 5' and 3' termini of *gnaeqD* in the three pAEQ1 clones were sequenced to ensure that digestion and ligation had inserted *gnaeqD* without errors. Manual sequencing using both

pNOM102 vector to produce the vector pAEQ1. Theoretically, the ligation inserted *gnaeqD* in the correct orientation under the control of its own start codon. Competent DH5α cells were transformed by electroporation using the ligation mixtures. Miniprepped transformants were screened by restriction analysis using *Hin*dIII, which would excise the *gnaeqD* insert and T*trp*C as a single fragment if *gnaeqD* was present, and would otherwise linearise the empty plasmid. Three successful clones, pAEQ1-1, pAEQ1-2 and pAEQ1-3 were chosen for further work.

Prior to production of the final vector for transformation, the 5' and 3' termini of *gnaeqD* in the three pAEQ1 clones were sequenced to ensure that digestion and ligation had inserted *gnaeqD* without errors. Manual sequencing using both guanidine and inosine bases showed all three to be correct up to 50 bp into *gnaeqD*. Therefore pAEQ1-1 was chosen to continue vector production.

To allow for selection of pAEQ1-1 in *Aspergillus*, an expression cassette containing *amdS* which encodes the acetamidase gene of *A. nidulans* was cloned into pAEQ1-1. *amdS* allows for selection of transformants that are capable of growing on acetamide or acrylamide as a nitrogen or carbon source (Kelly and Hynes, 1985). The *amdS* expression cassette was derived from the vector pAW4155S (supplied by TNO, The Netherlands), where the cassette was present as a 5.1 kb *Eco*RI fragment. pAEQ1-1 contains a unique *Eco*RI site upstream of the *gpdA* promoter (Figure 5.2) which allowed simple ligation of the *amdS* expression cassette into pAEQ1-1. This was done, with the 5.1 kb *Eco*RI fragment from pAW4155S being isolated by agarose gel electrophoresis. pAEQ1-1 was digested with *Eco*RI and phosphatased prior to ligation. DH5α cells were transformed with the ligation products by electroporation, and successful transformants selected on LB plates containing ampicillin. Ten transformants were picked, miniprepped and analysed by restriction digestion using *Eco*RI.

Two successful clones, pAEQ1-14 and pAEQ1-15 (Figure 5.3), were found by restriction analysis and these were used for transformation of *Aspergillus*.

5.3 *Aspergillus* Transformation

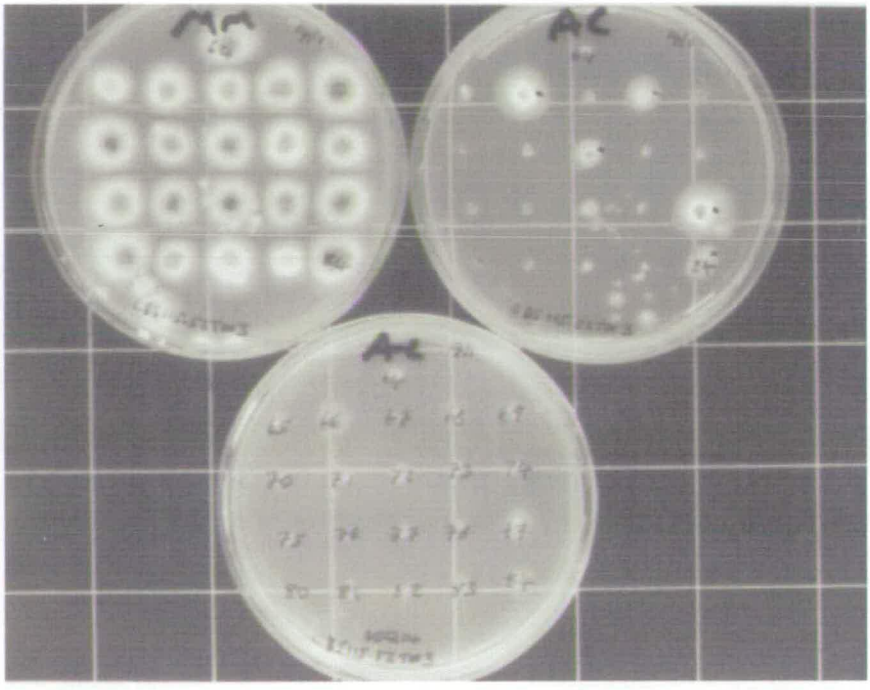
The method for *Aspergillus* transformation used protoplasting to remove the cell wall, and then the use of polyethyleneglycol (PEG) and $MgSO_4$ to permeabilise the plasma membrane and allow the transforming DNA to enter the cell. Transformation efficiency is very low compared to prokaryotic transformation, and therefore a large amount of DNA is required. Qiagen midi preps or maxi preps were used to bulk up the transforming DNA.

Two strains of *Aspergillus* were used for transformation; *A. niger* AB1.13 and *A. awamori* CBS115.52T#3. Both strains are knock-outs of the orotidine-5'-phosphate decarboxylase gene (*pryG*$^-$) and also lack the acetamidase gene of *A. nidulans* or any detectable homologue (Kelly and Hynes, 1985). Two methods of selection could therefore be used, which increases the stringency of the transformant selection, minimising the background of untransformed and abortive colonies. For *pyrG*$^+$ selection, the plasmid pAB4-1 (supplied by TNO, The Netherlands) was used, which contained the *pyrG* gene of *A. niger* (van Hartingsveldt *et al.*, 1987). *pyrG* is an auxotrophic marker, and therefore complementation selection used plates without the required nutrient, uridine, to select for positive transformants. The apoaequorin expression vectors contained *amdS* for selection using acetamide or acrylamide as a nitrogen source.

Each transformation was carried out using either 5 or 8 μg of both pAB4-1 and pAEQ1-14 or pAEQ1-15. Transformation mixtures were plated out as described in Section 2.10. *A. niger* transformants were selected using acrylamide and *A. awamori* transformants initially selected using acetamide. *amdS* selection is known to be

more stringent in *A. awamori* and growth on acetamide indicates multicopy presence of *amdS*. Picked *A. awamori* transformants were subsequently tested for growth on acrylamide. Numbers of viable protoplasts per transformation were determined by plating out control transformation dilutions on plates with and without osmotic protectant. A summary of the transformations carried out is given in Table 5.1 and the number of transformants obtained and analysed is given in Table 5.2.

Figure 5.4: Phenotypic comparison of picked transformants from Transformation series 5



Key; plates, from top left clockwise are; minimal media with uridine (no selection), minimal media with acetamide and minimal media with acrylamide. Colonies replica-plated in the same order on each plate

Table 5.1: Transformations of *Aspergillus* strains using pAEQ1-14 or pAEQ1-15

Transformation series	plasmids	strain	viable protoplasts/ transformation
1	pAEQ1-14 + pAB4-1	AB1.13	9×10^6
2	pAEQ1-15 + pAB4-1	AB1.13	9×10^6
3	pAEQ1-14 + pAB4-1	AB4.1	5×10^6
4	pAEQ1-15 + pAB4-1	AB4.1	5×10^6
5	pAEQ1-14 + pAB4-1	T#3	3×10^6
6	pAEQ1-15 + pAB4-1	T#3	9×10^5
7	pAEQ1-14 + pAB4-1	T#3	9×10^5

Table 5.2: *Aspergillus* transformation results with pAEQ1-14 or pAEQ1-15

Transfor-mation series	colonies (mean per transformation)	µg of each plasmid	mean transformation freq/ µg DNA	mean transformation freq/ µg DNA/ 10^7 protoplasts	No. colonies picked
1	26.6	5	5.32	5.9	21
2	18	5	3.6	4.0	21
3	9.8	5	1.96	3.92	10
4	10.2	5	2.04	4.08	10
5	69.33 (15.04)	8	8.7 (1.9)	28.2 (1.7)	83 (18)
6	2.75	5	0.55	6.1	7
7	3.5	5	0.7	7.8	4

Key to Table 5.1 and Table 5.2: each transformation series involved 4 or 5 individual transformations under the same conditions. Viable protoplasts/ transformation and number of colonies obtained refer to individual transformations. The number of colonies represents only strong, sporulating colonies after 3 days incubation at 30 °C. Mean transformation frequencies have taken the average number of colonies obtained from the individual transformations within each transformation series. Figures for transformation 5 indicate the results obtained by initial selection for *pyrG*$^+$ only, the numbers in parentheses representing the results obtained after selection for *pyrG*$^+$ and *amdS*.

As shown in Tables 5.1 and 5.2, a large number of transformants were obtained for *A. niger* and for *A. awamori*. However, in all cases the transformation frequency was low in comparison to some of the published transformation frequencies. Transformation frequencies of 40-100 transformants per µg DNA for *A. niger* have previously been obtained (Goossen *et al.*, 1987; van Gorcom and van den Hondel, 1988; van Hartingsveldt *et al.*, 1987). Variation in transformation frequency is expected between different strains, and it was also noticed that the transformation

frequency dropped markedly if the DNA used was not very clean. A higher transformation frequency was obtained when Qiagen midi prep or maxi prep DNA was used that had been subjected to a second isopropanol precipitation. Only the strongest growing colonies were counted and picked (Table 5.2) for further phenotypic comparison. This was done by inoculating each onto rich media with glucose, and also double selection plates for *pyrG*$^+$ and *amdS*, using either acetamide or acrylamide for *amdS* selection (see Section 2.2.1). This allowed comparison of the growth strength of the individual transformants against one another and against the untransformed original strain. An example of such plates for transformants selected from transformation series 5 is shown in Figure 5.4. As can be seen, the difference in growth rate on the different media is pronounced. This is increased in *A. awamori* compared to *A. niger*, as *A. awamori* is more sensitive to the use of acrylamide for selection. It is widely accepted that a colonies growth strength on selective media represents the expression level of the transformed selection genes (Kelly and Hynes, 1985). Since transformation generally inserts the transforming DNA as tandem repeats, it is expected that if the selection genes are expressed highly then the desired heterologous gene is also in a favourable position in the genome for expression. Strength of growth is indicated by the rate of growth (i.e. colony size) and sporulation density.

From such comparisons, the strongest colonies were chosen for purification. Purification was required as transformed protoplasts were expected to be multinucleate, and it is very unlikely that the transforming DNA would be incorporated into every nucleus, and if it was, each nucleus would have a different copy number and integration site. Transformants were purified using two rounds of serial isolation of colonies on selective media. Purified transformants were then analysed for apoaequorin expression levels and further phenotypic comparison with the parent strain.

5.4 Transformant screening

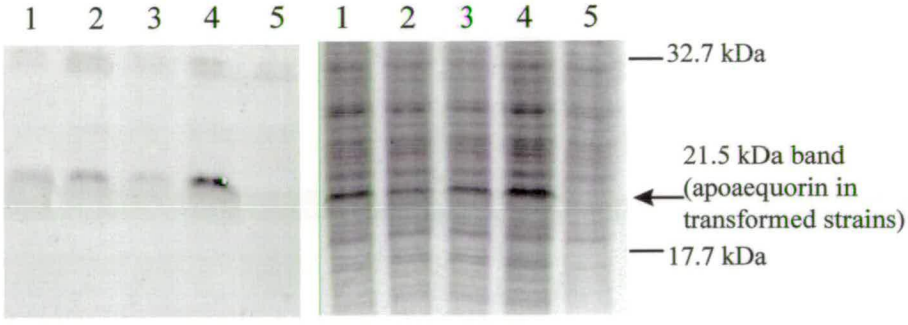
5.4.1 Production of mouse anti-apoaequorin antisera

Purified His-tagged apoaequorin (Section 4.5.2) was used to raise polyclonal mouse anti-aequorin antibodies. This was carried out in the Institute of Cell and Animal Population Biology, University of Edinburgh, using 10 MF-1 outbred mice.

5.4.2 Western blotting

Initial analysis of apoaequorin expression levels in transformants of transformation series 1 (Table 5.1) was carried out by Western analysis of soluble whole cell extracts using polyclonal mouse anti-aequorin antisera and BCIP to visualise apoaequorin. The strongest growing colonies were used for comparison. Samples were electrophoresed through a 12.5% polyacrylamide gel. A small amount of background binding was seen in the Western, but a band of the correct size (approx. 21.5 kDa) was seen in the purified transformants that was absent from the control lane (Figure 5.5). This was visible on the Coomassie-stained gel, and the bands were confirmed on the Western blot (Figure 5.5).

Figure 5.5: Coomassie-stained SDS-PAGE and Western blot of whole cell extracts from *A. niger* AB1.13 transformants



Key: Western shown on the left, Coomassie-stained gel shown on the right. Well numbers as follows: 1- AB1.13AEQ14-1; 2- AEQ14-2; 3- AEQ15-1; 4- AEQ15-21; 5- AB1.13 untransformed

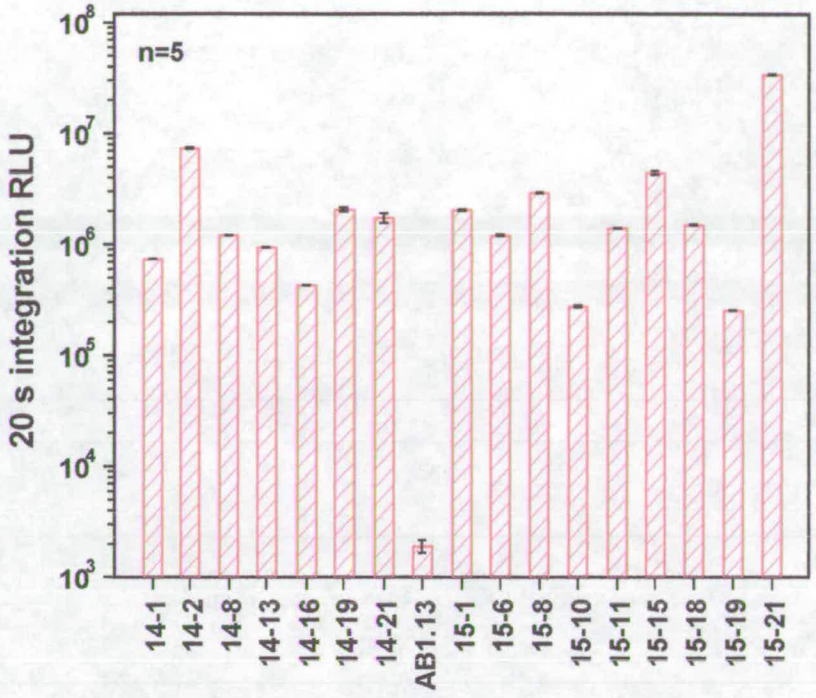
Western analysis does not predict whether the apoaequorin was active, and it is noticeable that the intensity of the bands observed in the Coomassie-stained gel does not correlate with the intensity of the bands obtained in the Western. This is especially noticeable in lanes 1 and 3. Since the Western was carried out on a different gel to that of the Coomassie-stained gel, then it is possible that there were differences in the amount of protein loaded. The apoaequorin band intensities varied between transformants, and subsequent analysis by luminometry confirmed the variations in aequorin production. Also, the band intensities correlated with the strength of growth of the transformants on selective media, with transformant 15-21 being the strongest growing colony and giving the most intense band. This matches previous observations on transformant growth and expression levels (Kelly and Hynes, 1985). There is a slight variation in loading concentrations, with lane 4 being loaded more concentrated than the other lanes. This should be taken into account when comparing the band intensities on the Western. As can be seen in Figure 5.5, there are no discernible differences between the whole cell extracts from the transformants compared to the untransformed control. This suggests that no major disruption has occurred at recombination.

5.4.3 Luminometry

Luminometry analysis was initially performed using protoplasts, following a method developed by Collis (1996). As a standard comparison of apoaequorin expression, known quantities of protoplasts were treated with 100 mM $CaCl_2$ to release constituted aequorin luminescence by the presence of an excess of Ca^{2+} ions. Luminometry was performed throughout, and the results after addition of Ca^{2+} integrated over 20 s. Such data for *A. niger* AB1.13 purified transformants is shown in Figure 5.6.

The method using protoplasts was altered after the observation that it was possible to measure luminescence from growing hyphae that had been incubated in the presence of coelenterazine. Previously, this had not been possible in *N. crassa*, probably due to the low levels of apoaequorin produced making it impossible to have enough mycelial density in the luminometer (Collis, 1996). The method developed for luminometry *in vivo* of growing young mycelia is outlined in Section 2.2.1.2.3.3. This was subsequently used for comparison of purified transformant strains. The method used involved adding 100 mM $CaCl_2$ and monitoring luminescence. As previously, a sharp increase in cytoplasmic Ca^{2+} was seen, and this was integrated over 20 s to compare luminescence between different transformants. An example of such data is shown in Figure 5.7 for the analysis of *A. awamori* CBS115.52 T#3 transformants.

Figure 5.6: Integrated bioluminescence from protoplasts expressing aequorin

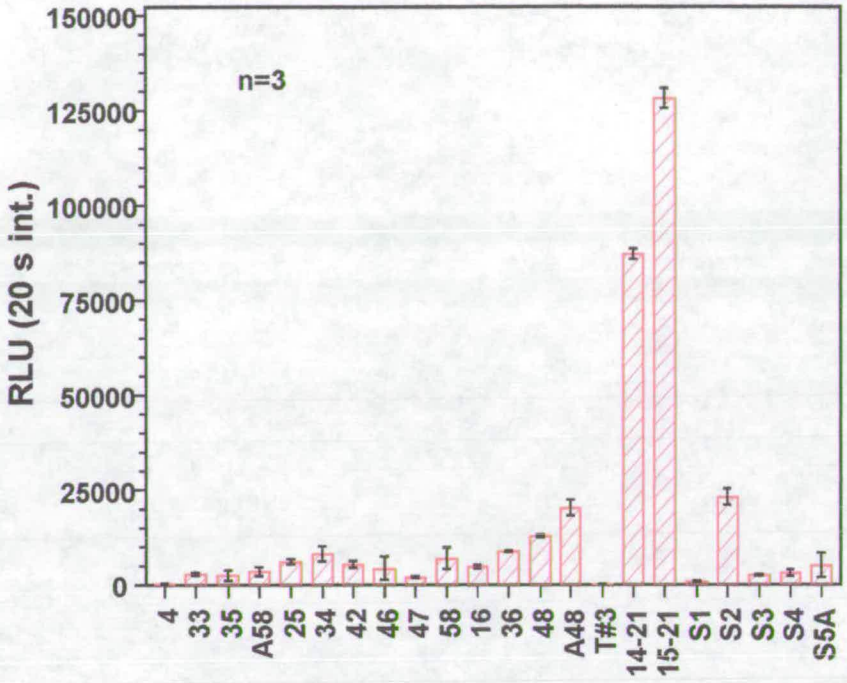


Key: bioluminescence integrated over 20 s after stimulation with 100 mM $CaCl_2$ in 1 M sorbitol. 1 x 10^6 protoplasts per sample. x- axis shows transformant numbers. AB1.13 represents the untransformed parent strain. n=5, error bars are SD(n-1).

The protoplast aequorin expression levels shown in Figure 5.6 are comparable with the density of bands seen in the Western blot (Figure 5.5).

From the luminometry assays of strains (not all data shown), several high expression transformants were chosen for comparison against one another. One transformant of *A. niger* AB1.13, and five transformants of *A. awamori* CBS115.52T#3 were compared, using *in vivo* luminometry of young mycelia as described previously. The data is shown in Figure 5.8. From this data, two high expression transformants were chosen for further work. These were *A. niger* AB1.13:AEQ15-21 and *A. awamori* CBS115.52 T#3:66A (referred to as strains 15-21 and 66A respectively).

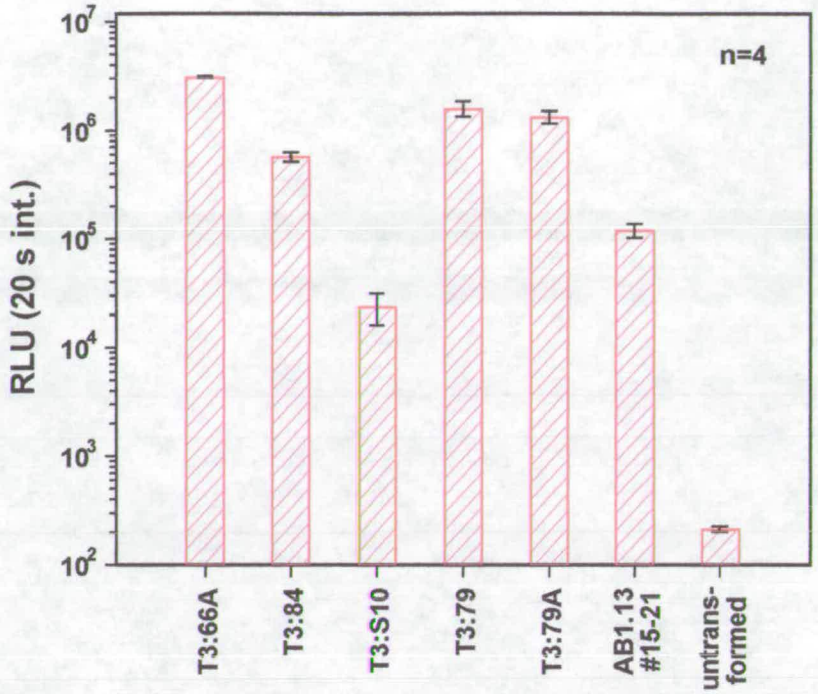
Figure 5.7: Integrated bioluminescence from *A. awamori* transformants



Key: bioluminescence integrated over 20 s after stimulation with 100 mM $CaCl_2$. Spore density at inoculation; 5,000 spores per sample. x- axis shows transformant numbers. T#3 represents the untransformed parent strain, *A. awamori* CBS115.52T#3. 14-21 and 15-21 are two transformants of *A. niger* AB1.13 and are shown for comparison to Figure 5.6. n=3, error bars are SD(n-1).

It is noticeable that the data replication is very consistent for each transformant. Very small differences were observed due to variations in the rate of growth between one set of samples and another set of the same transformant strain. This was presumably due to fluctuations in the incubator temperature, as everything else was kept constant, and pipetting error would be seen within a set of samples.

Figure 5.8: Integrated bioluminescence of high expression transformants

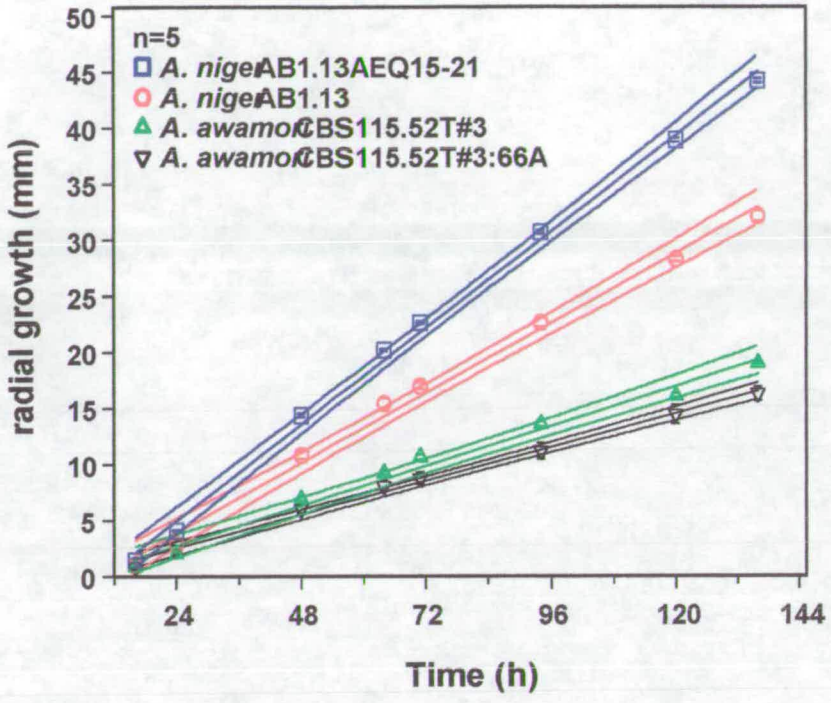


Key: see Figure 5.7. spore density at inoculation = 5,000 per sample. 'untransformed' represents the parent strain *A. awamori* CBS115.52T#3. 15-21 is a high expression transformant of *A. niger* AB1.13. Other transformants shown are derived from *A. awamori* CBS115.52T#3. n=4, error bars are SD(n-1).

5.4.4 Comparison of growth rates

As a phenotypic comparison to the parental strains, 15-21, 66A and their parent strains growth rates were measured on 25 ml RMG agarose plates (9 cm diameter, with uridine added for parent strains) at 27 °C as shown in Figure 5.9.

Figure 5.9: Growth comparison between transformants and parent strains



Key: curves plotted as linear regression with 95% confidence limits. Error bars [SD(n-1)] are too small to be visible.

There is a small difference in growth rates between the transformant strains and the parent strains. It is noticeable that 15-21 grows faster than the parent strain, AB1.13. This may be due to the parent strain being an auxotroph, yet the same is true for the *A. awamori* strains, and the transformant grows slower than the parent strain. Site of integration at transformation probably plays a major role in the growth rate differences due to perturbed gene expression or differences in levels of transgene expression for the *pyrG* marker, which is required for growth. Another possible explanation is that *pyrG* is under the control of an *A. niger* promoter, and therefore would be expected to be more strongly expressed in *A. niger* than in *A. awamori*, which would account for the better growth of the *A. niger* transformant.

It was observed that the timing of conidiation and the density of conidia produced were the same for the transformants and the parent strains.

It can be concluded that the presence of a large amount of apoaequorin in the transformants appears to make little difference to the phentoype of the transformants as compared to the parental strains.

5.5 Calibration of aequorin concentration

Using the standard curve obtained for aequorin concentration (Figure 4.9), soluble cell extracts from *Aspergillus* transformants 66A and 15-21 were analysed *in vitro* to estimate their total aequorin concentrations. Protein extraction and analysis was carried out as described for *N. crassa* (Section 4.4). The results are shown in Table 5.3. The levels of aequorin present are far higher than those found in *N. crassa* transformants; the best *Neurospora* transformant producing 22.6 *p*moles aequorin/ µg protein (Table 4.2). This is over 200 times lower than the amount produced in *A. awamori* 66A.

Table 5.3: Comparison of aequorin concentrations in soluble whole cell extracts of *Aspergillus*

Transformant strain	RLU (20 s integration after stimulation)	*f*moles aequorin per 10 µg protein extract	*n*g aequorin/ µg protein ±SD
A. niger 15-21	6,443,998 ± 148,703	1370.7	3.02 ± 0.16
A. awamori 66A	7,926,260 ± 134,343	2220.5	4.89 ± 0.19

Key: RLU shows the mean RLU ±SD obtained over 20 s after stimulation with 100mM $CaCl_2$, with the mean control background count subtracted. *f*moles aequorin represented as means of 6 samples. 1 *f*mole aequorin weighs 2.2×10^{-11}g. A Student's *t*-test assuming unequal variances gave a *p* value of 7.404×10^{-9}, showing difference between the two strains is very highly significant.

5.6 Discussion

The results detailed in this chapter describe the production of a fungal expression vector for the synthetic apoaequorin gene, and the subsequent transformation of

Aspergillus species with the vector. Successful transformants have been isolated and from these, two transformants, AB1.13:AEQ15-21 and CBS115.52T#3:66A, have been selected to be used for physiological studies. Comparisons of phenotype by growth rate, morphology and soluble protein bands (for 15-21) suggests that 15-21 and 66A are as near to the phenotype of their respective parent strains as can be expected.

The transformation efficiencies obtained appear to be low compared to published transformation efficiencies. This may be due to the use of double selection for transformants. This was highlighted in transformation series 5 (Table 5.2), where only *pyrG*$^{+}$ selection was used for the initial transformants. This series of transformations produced an initially high frequency of transformation (28.2/μg DNA), which upon double selection of the initial positive transformants reduced their numbers almost five-fold, as the representative sample shown in Figure 5.4 demonstrate. The published results for the development of *pyrG* as a selectable marker reported a frequency of 40 transformants per μg DNA for between 0.1-1 x 10^7 protoplasts (van Hartingsveldt *et al.*, 1987). Another factor which may have hindered transformation frequency may have been use of DNA which was not clean enough and contained too much salt. This is unlikely due to the extra wash steps that were carried out on the DNA after production.

6. CALCIUM MEASUREMENT IN *ASPERGILLUS*

6.1 Introduction

As outlined in Chapter One, modulation of $[Ca^{2+}]_c$ is known to occur in filamentous fungi in response to a variety of stimuli. As described in the previous chapter, transformants of *Aspergillus niger* and *A. awamori* expressing high levels of aequorin were produced. These transformants allow the simple measurement and monitoring of $[Ca^{2+}]_c$ in these fungi.

Prior to using the transformants for analysis of *in vivo* responses to different external stimuli, several control conditions were determined. Variable factors which had to be resolved were conditions such as coelenterazine concentration and autoluminescence, and the time constraints for using growing cultures. Once control of these variables had been determined, it was possible to use the cultures under standardised conditions for monitoring $[Ca^{2+}]_c$. The effects on $[Ca^{2+}]_c$ of a range of external stimuli were investigated, including: the addition of external calcium, mechanical perturbation, osmotic shock, changes in external pH and the addition of antifungal proteins. All data represented in this chapter is from the *A. awamori* transformant 66A (Section 5.4.2) unless otherwise stated. Many of the experiments were carried out with both this strain and *A. niger* transformant 15-21, and the same results were obtained for both. Eventually, only *A. awamori* 66A was used, as this gave a higher level of aequorin expression (Section 5.4.2). Unless otherwise stated, the method of culture growth and coelenterazine incubation was as described in Section 2.2.1.2.3.3.

The work shown in this chapter represents a preliminary investigation into $[Ca^{2+}]_c$ signalling in *Aspergillus* using recombinant aequorin. The main aim was to evaluate

the recombinant aequorin technique in *Aspergillus* as a simple and routine method for measuring $[Ca^{2+}]_c$ dynamics in living hyphae.

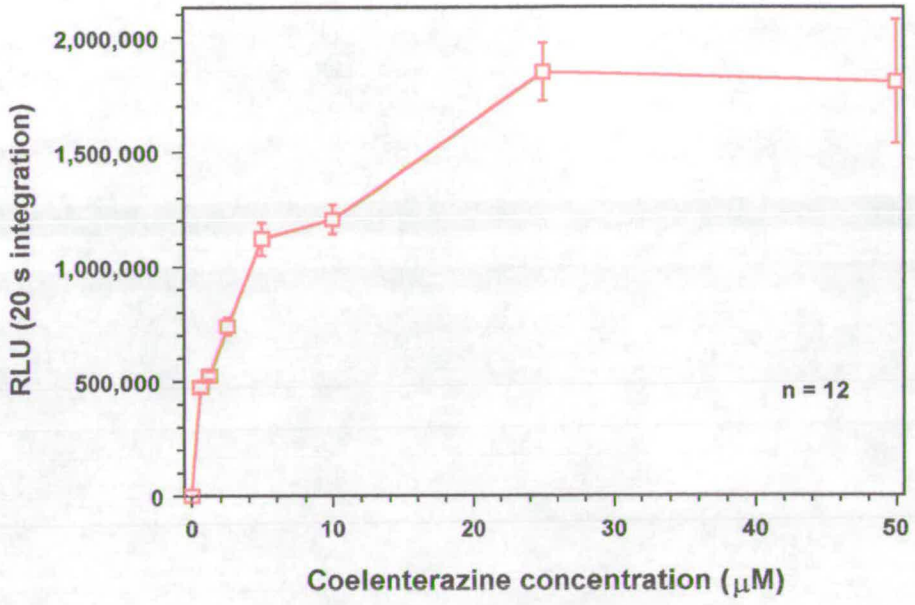
6.2 Optimisation of parameters for aequorin formation

6.2.1 Influence of coelenterazine concentration on aequorin reconstitution

Previous attempts to monitor aequorin luminescence in living cells of a filamentous fungus have been largely unsuccessful due to the low levels of apoaequorin expression. It was found to be possible to monitor luminescence in *N. crassa* only using a large number (5×10^6) of protoplasts which had been incubated with a high concentration (50 μM) of coelenterazine (Collis, 1996). Use of coelenterazine at such concentrations has drawbacks, such as expense and autoluminescence. Therefore the influence of coelenterazine concentration upon reconstitution of active aequorin *in vivo* in *Aspergillus* was investigated. Eighteen hour multi-well cultures in VS medium (as described in Section 2.2.1.2.3.3) were incubated with varying concentrations of coelenterazine for four hours, prior to stimulation with a high concentration (100 mM) of external Ca^{2+} (Figure 6.1), which had been found to elicit a large and reproducible increase in $[Ca^{2+}]_c$ (Section 6.3.3).

The luminescence response to external Ca^{2+} increases up to a concentration of approx. 30 μM coelenterazine (Figure 6.1). The light levels obtained with a low coelenterazine concentration (2.5 μM to 5 μM) were high enough to be used routinely for $[Ca^{2+}]_c$ measurement in *A. awamori*.

Figure 6.1: Influence of coelenterazine concentration on the formation of active aequorin in living hyphae of *A. awamori* 66A

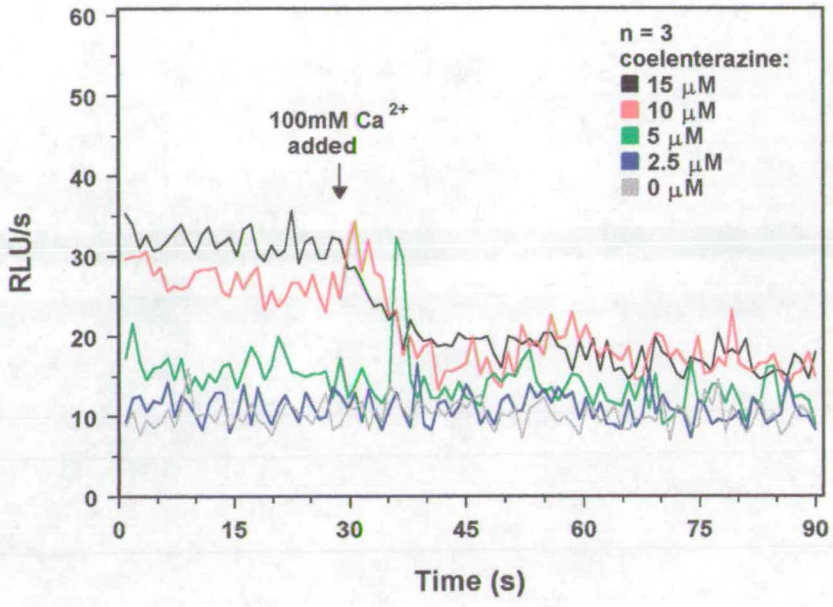


Key: y-axis represents 20 s integrated luminescence after treatment with 100 mM $CaCl_2$ (final Ca^{2+} concentration 50 mM). Coelenterazine dissolved in 100% MeOH and dilution series made using VS medium (final [MeOH] never exceeded 0.1% v/v). Wells incubated in coelenterazine for 4 h. n = 12, error bars are ±SD.

6.2.2 Coelenterazine luminescence

Coelenterazine is slightly luminescent, to an extent that can be detected at high concentrations using a luminometer (Collis, 1996). A range of concentrations of coelenterazine were incubated in VS medium for 4 h and the background luminescence before and after the addition of Ca^{2+} monitored (Figure 6.2). There was noticeable luminescence (2.5 - 3.5 times the control background) of coelenterazine in VS medium at concentrations of 10 and 15 μM. Although Figure 6.2 does not show the error bars for clarity of the data, using a one way ANOVA and calculating the 5% LSD showed that all concentrations of coelenterazine generated luminescence levels which were significantly different from one another prior to addition of Ca^{2+} (Table 6.1).

Figure 6.2: Luminescence of coelenterazine in VS medium



Key: background monitored for 30 s prior to addition of 100 mM $CaCl_2$ at $t = 30$ s. n = 3. Error bars not shown for clarity. Statistical analysis shown in Table 6.1.

Table 6.1: Statistical analysis of coelenterazine luminescence

Coelenterazine concentration (μM)	Mean RLU/s over 20 s period
15	31.72727
10	26.9899
5	15.91919
2.5	11.33333
0	10.13131

Key: Mean RLU/s calculated from data shown in Figure 6.2, from $t = 0$ to 20 s. The 5% LSD was calculated as 0.909, which shows that the mean luminescence for each concentration of coelenterazine were significantly different from one another.

Coelenterazine luminescence was quenched when Ca^{2+} was added to the samples. Measurement of low levels of luminescence was required to allow monitoring of background $[Ca^{2+}]_c$ in growing hyphae. Therefore it was concluded that a concentration greater than 5 μM coelenterazine was undesirable in VS medium, as

this increases the background noise. Combined with the data shown in Figure 6.1, a coelenterazine concentration of 2.5 μM was chosen for future work.

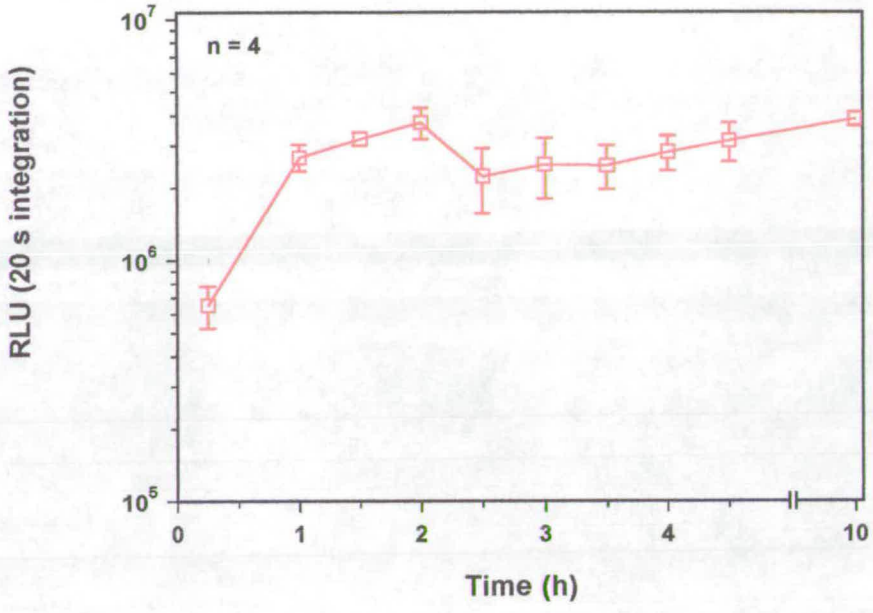
Other media were analysed for their effect upon coelenterazine luminescence (data not shown), including minimal media with glucose (Section 2.2.1.1.1) and RMG (Section 2.2.1.1.3). RMG gave a very high background of luminescence, and this was identified as being due to the presence of yeast extract in the medium. Minimal media with glucose gave the same results as those obtained with VS (Figure 6.2). From previous studies, the autoluminescence could be minimised by using HPLC-grade H_2O as the medium (Collis, 1996). However, to monitor $[Ca^{2+}]_c$ during growth the cultures must be kept in growth medium, and therefore use of HPLC H_2O as a medium was not possible.

6.2.3 Rate of coelenterazine uptake and aequorin formation

The rate of coelenterazine uptake by hyphae and the formation of active aequorin was measured using 18 h cultures incubated with 2.5 μM coelenterazine. Active aequorin was measured by treating wells with 100 mM $CaCl_2$ at different intervals after coelenterazine addition (Figure 6.3).

Coelenterazine uptake and active aequorin formation appeared to be very fast because there is little difference in luminescence after 2.5 h post-coelenterazine addition. It is not known why there is a drop of approx. one third in luminescence between 2 h and 2.5 h, which then recovered over the next 2 h. This trend was found to be repeatable. To avoid such variation, cultures were analysed after a coelenterazine incubation of 3 h, unless otherwise stated.

Figure 6.3: Influence of period of coelenterazine incubation on active aequorin formation in hyphae

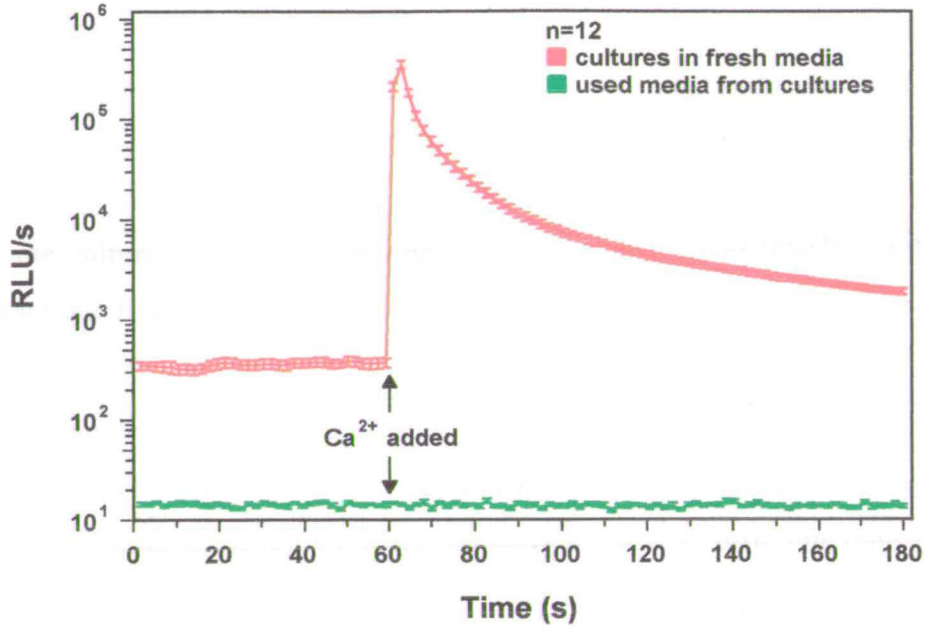


Key: x-axis represents time of incubation with coelenterazine. y-axis represents the integrated luminescence obtained over 20 s after addition of 100 mM $CaCl_2$ (final Ca^{2+} concentration 50 mM). Control background RLU measurements were negligible (< 20 RLU/s for all points). n = 4, error bars are ± SD.

To verify that active aequorin was not exported from hyphae into the media of cultures, 24 h *A. awamori* cultures were incubated with 2.5 μM coelenterazine for 3 h and then resuspended in fresh media. Active aequorin in the spent media and cultures was then stimulated with the addition of 100 mM $CaCl_2$ (Figure 6.4).

There was no luminescence response in the spent media of *A. awamori* 66A cultures, and the light levels obtained were similar to a typical background count (*e.g.* see Figure 6.2), which suggests that active aequorin is not released into the media. However, it is possible that any released aequorin will have been immediately discharged in the media and therefore not recorded in this experiment, as VS medium contains 0.68 μM $CaCl_2$. The response shown by the mycelia to the addition of $CaCl_2$ is the standard response obtained with this stimulus (Section 6.3.3).

Figure 6.4: Ca^{2+} response of young hyphae and spent media of *A. awamori* 66A.

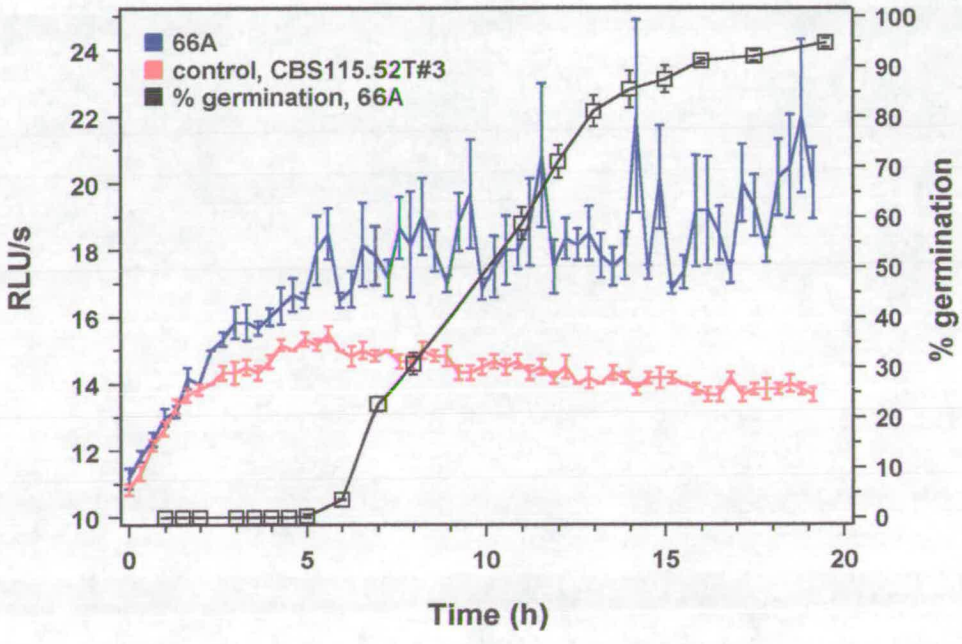


Key: 24 h cultures used. 100 mM $CaCl_2$ added (final Ca^{2+} concentration 50 mM) at t = 60 s. n = 12, error bars are ±SD.

Since the response to external Ca^{2+} is very rapid, it is likely that most of the active aequorin is present in the cytosol and not compartmentalised within organelles. If aequorin was present in organelles, then the response would be expected to be slower. Also, if aequorin was present in Ca^{2+} storing organelles such as the vacuole, then the background would be expected to be higher. The response signature would also be expected to be different, depending upon whether the aequorin-containing store was utilised in the response to external Ca^{2+}. It is probable that aequorin is present within the nucleus, as nuclear pores are permeable to 21.5 kDa proteins. The background luminescence observed for the spent media was of the same level as both control unused media and spent media from the untransformed parent strain, *A. awamori* CBS115.52T#3. The same observation was obtained for *A. niger* 15-21 (data not shown).

transformant, 66A (a Student's *t*-test of the average luminescence from each well between 10.5 h and 19.5 h produces a *p* value of 6.08×10^{-5} between the two groups of data).

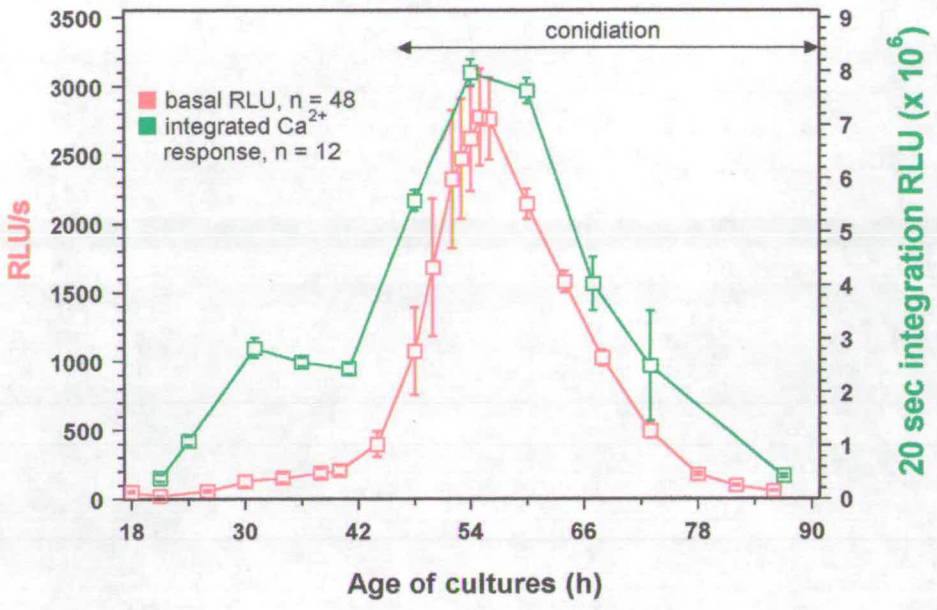
Figure 6.5: Germination rate and luminescence of *A. awamori* germlings



Key: Average percentage germination rate taken from percentage germination seen in 3 samples from 3 wells for each point. Germination rate (black line) measured on right hand y-axis. Error bars are ±SEM. Basal luminescence (blue and red lines) measured on left hand y-axis. For luminescence measurements, n = 6, error bars are ±SD.

Basal luminescence measurements of cultures from 18 h onwards, once germination had reached its maximum, followed the growth of mycelia to produce a mat in the well and subsequent conidiation (Figure 6.6). Since *A. niger* and *A. awamori* conidia are black and dark brown respectively, it can be assumed that once conidiation becomes very dense, very little luminescence will be visible from the top of the well, where the photon multiplier tube is positioned. The basal level of luminescence was monitored for 70 h after addition of coelenterazine to 18 h cultures, and the response to external Ca^{2+} was also monitored over this period (Figure 6.6).

Figure 6.6: Long term growth of *A. awamori* 66A as measured by luminescence



Key: x-axis denotes time (h) after addition of coelenterazine to 18 h cultures. Basal luminescence (red line) measured on left hand y-axis. n = 48, error bars are ±SD. Response of cultures to the addition of 100 mM $CaCl_2$ (green line) is measured as a 20 s integration of luminescence after stimulation, and is shown on the right hand y-axis. n=12, error bars are ±SD. An indication of the timing of conidiation in the samples is given by the arrow denoted 'conidiation'.

Analysis of the basal luminescence shown in Figure 6.6 showed that the luminescence obtained increased with growth of the mycelia for 24 h (18 to 42 h cultures), after which the basal luminescence increased significantly at the onset of conidiation. There is a greater variation in the data obtained over this period due to variation in the timing of conidiation between different wells. Culture biomass changed very little during conidiation (data not shown), which suggests that the increase in basal luminescence is correlated with the onset of conidiation. The rate of increase in the basal luminescence level does not correlate with the growth rate of the strain, and would be expected to decrease with the increasing density of conidia blocking the amount of light that could reach the top of the well. The background noise, as measured by growth of untransformed *A. awamori* showed a consistently low signal below 20 RLU/s, which is too low to be shown on Figure 6.6 but is shown on Figure 6.5.

The data obtained for the mycelial response to external Ca^{2+} during conidiation suggests that the increase is due to either an increase in the levels of aequorin present in the cultures or heightened sensitivity to the addition of Ca^{2+} to the media. Combined with the findings for the basal luminescence, it suggests that the former is the case, as a heightened sensitivity to external Ca^{2+} would not be expected to give an increase in the basal luminescence. The increase in basal luminescence seen could also be accounted for by the presence of a higher $[Ca^{2+}]_c$, however it is difficult to explain why the response to external Ca^{2+} should also increase if this was the case. Analysis of aequorin concentration in soluble whole cell extract from cultures of different ages would verify whether aequorin concentration increases during conidiation. This was not carried out in this study. An increase in the levels of aequorin produced could possibly be due to the choice of promoter, although why the *gpdA* promoter is stimulated to such high levels during conidiation is unknown. The subsequent drop in activity is thought to be due to the dense conidiation, which in turn is due to starvation of resources for the culture, which will also play a part in limiting protein (and hence aequorin) production.

Both Figure 6.5 and Figure 6.6 show that addition of 2.5 μM coelenterazine to the wells provides a large excess of coelenterazine which lasts a long time, and is therefore quite stable in the medium. However, degradation of coelenterazine or lack of sufficient coelenterazine may play a part in the decreased luminescence observed in Figure 6.6 40 h after coelenterazine addition, although as stated above, this would make little difference due to the density of conidiation. Analysis of aequorin activity during conidiation was not investigated further due to the problems of conidiation and their dispersal in the lab.

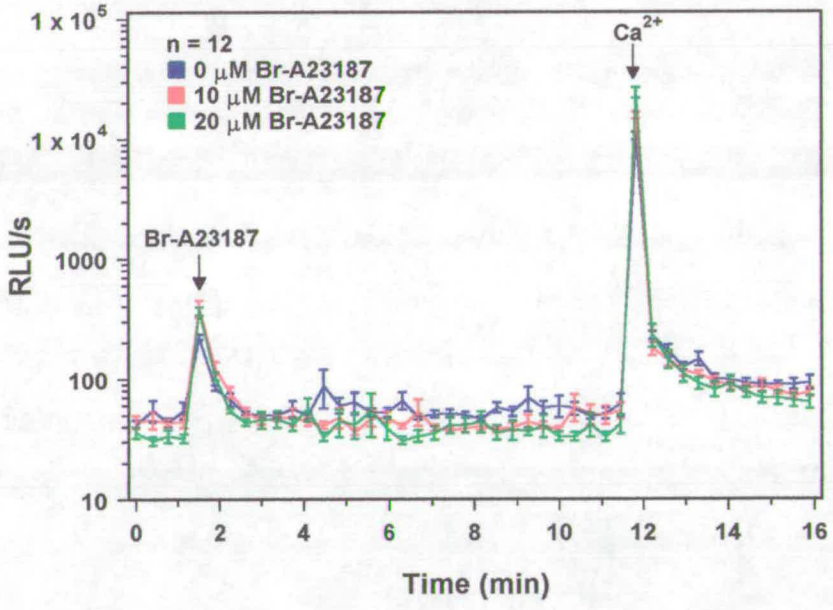
Subsequent studies used cultures which were between 18 and 36 h old, generally 24 h cultures, during which time there is steady growth of the mycelia with a concomitant increase in aequorin. The stimuli investigated were not seen to produce different responses over this period of growth.

6.3 Response to external calcium

6.3.1 Effect of ionophore Br-A23187

The ionophores A23187 and 4-bromo-A23187 (Br-A23187) allows rapid equilibration of Ca^{2+} across cell membranes (Deber and Hsu, 1986). It forms a liposoluble complex with Ca^{2+} which causes an increase in cell membrane permeability for Ca^{2+}. A23187 has been used previously with transgenic aequorin in *S. cerevisiae* to show a rapid increase in luminescence (Nakajima-Shimada *et al.*, 1991) upon addition with 20 mM $CaCl_2$. In contrast however, use of Br-A23187 with aequorin transformed *N. crassa* protoplasts showed no difference in response between stimulation with $CaCl_2$ and $CaCl_2$ with ionophore (Collis, 1996).

Figure 6.7: Effect of Br-A23187 upon *A. awamori* response to external Ca^{2+}



Key: 18 h cultures used. Br-A23187 dissolved in DMSO at a concentration of 10 mM. 100 µl VS + DMSO or Br-A23817 added at t = 1 min 9 s (final DMSO concentration ≤0.004% v/v). 100 µl 100 mM $CaCl_2$ added at t = 11 min 46 s (final Ca^{2+} concentration 33.3 mM). n = 12, error bars are ±SD.

Br-A23187 was added to *A. awamori* mycelia whilst monitoring luminescence, and the cells subsequently treated with 100 mM $CaCl_2$ (Figure 6.7).

Statistical analysis of the responses to addition of media (± ionophore) and the subsequent addition of Ca^{2+} was carried out (Figure 6.8). This showed that there is a statistically significant increase in the luminescence response in the presence of Br-A23187 for both the addition of Br-A23187 in media and after addition of Ca^{2+} compared to the control.

Analysis of the mean basal light level, both before addition of VS ±Br-A23187 (t = 0 to 1 min) and afterwards (t = 6 to 10 min) using a two factor ANOVA with replication showed no significant difference between treatments and before or after addition of VS ±Br-A23187. This suggests that at the concentration that Ca^{2+} is present in the media (0.68 mM), and at the concentrations at which Br-A23187 was used, there is a negligible effect of the ionophore upon resting $[Ca^{2+}]_c$. It is only upon external stimulation that there is any great increase in luminescence. Also, it can be seen from Figure 6.7 that Br-A23187 does not affect the recovery from either of the responses analysed.

Figure 6.8: Tabular representation of significance of responses shown in Figure 6.7

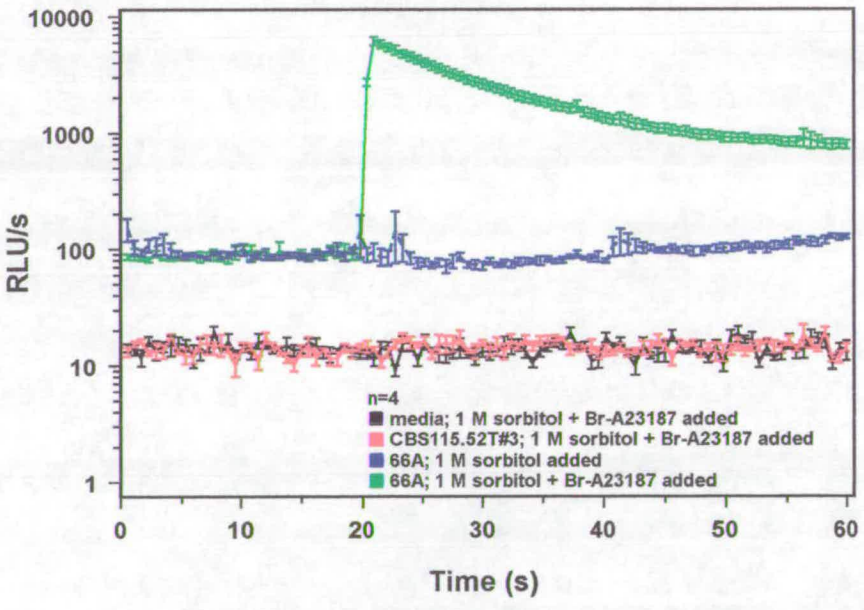
Stimulus	**Ionophore concentration**			**5% LSD**
	0 μM	10 μM	20 μM	
VS (± ionophore)	224.17	389.25	368.25	119.16
Ca^{2+} injection	10629.83	15685.67	24448.5	4764.64

Key: Analysis carried out using single factor ANOVA and calculating the 5% LSD. Means of peak responses (RLU/s) shown after the stated stimulus for each treatment, as shown in Figure 6.7. Underlined figures represent datasets that are not significantly different from one another.

The effect of Br-A23187 upon mycelial $[Ca^{2+}]_c$ appears to be slight when compared to published cytosolic responses to ionophore (Nakajima-Shimada *et al.*, 1991). This may be due to the presence of the cell wall diminishing the effect of the ionophore upon the cell membrane by allowing the cell more protection against its environment. To investigate this, the response of *Aspergillus* protoplasts to addition

of Br-A23187 was monitored (Figure 6.9). As can be seen, there is a very large response to the addition of 20 μM Br-A23187, which supports the theory of the cell wall inhibiting the effects of the ionophore. It must be noted that protoplasts are fairly compromised cells, and rely solely upon osmotic protection from the media, and therefore may be expected to be more susceptible to any change in their environment. However, the control transformant wells injected with iso-osmotic media (1 M sorbitol + DMSO) showed no response to the stimulus, and therefore the protoplasts produced can be assumed to be intact. Interestingly, the lack of response upon mechanical stimulation with 1 M sorbitol (+ DMSO) is unusual in comparison with intact mycelia, which produce a response upon stimulation with iso-osmotic media (Figure 6.7 and Section 6.4).

Figure 6.9: Response of *A. awamori* protoplasts to Br-A23187



Key: 5 x 10^5 protoplasts per well in 100 μl 1 M sorbitol. Wells incubated with 2.5 μM coelenterazine for 4 h prior to measurement. Control wells contained either 1 M sorbitol + coelenterazine (designated 'media' in figure) or untransformed *A. awamori* parent strain (CBS115.52T#3) + coelenterazine. Transformant wells (66A) injected with either 1 M sorbitol + 20 μM Br-A23187 or 1 M sorbitol + DMSO (0.004% v/v). n = 4, error bars are ±SD.

6.3.2 *Effect of external Ca^{2+} chelator EGTA*

The addition of a cell impermeable Ca^{2+} chelator diminishes the concentration of external Ca^{2+}. Ethyleneglycol-*bis*-(β-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA) is such a chelator which is selective for Ca^{2+}. Addition of EGTA to young cultures caused a decrease in basal luminescence, presumably due to perturbation of the electrochemical gradient by removal of free Ca^{2+} in the medium causing cytosolic Ca^{2+} to move into the medium (Table 6.2).

Table 6.2: Inhibition of luminescence due to addition of EGTA to *A. awamori* cultures

EGTA concentration added	mean RLU/s before addition (± SD)	mean RLU/s after addition (± SD)	*p* value
50 mM	32.01 ± 0.72	19.72 ± 0.26	6.64×10^{-17}
500 mM	47.14 ± 1.65	27.47 ± 0.46	9.88×10^{-18}
untransformed control:			
500 mM	14.03 ± 0.33	18.85 ± 2.28	0.12

Key: n = 12. Concentration of EGTA presented is concentration added to wells which dilutes 50% with the present media (therefore final concentrations were 25 mM and 250 mM). *p* values obtained for each pair of measurements using Student's *t* test (unequal variances). Analysis using a two factor ANOVA showed there to be a significant difference between the two treatments (50 mM and 500 mM EGTA). There is no significant difference in the luminescence of the control untransformed cultures.

Measurement of luminescence 10 min after addition of EGTA showed that $[Ca^{2+}]_c$ increased back to the original basal level (data not shown). The origin of the Ca^{2+} (whether from external or internal stores) for this recovery was not analysed, but is likely to be from internal stores since external Ca^{2+} has been chelated by EGTA.

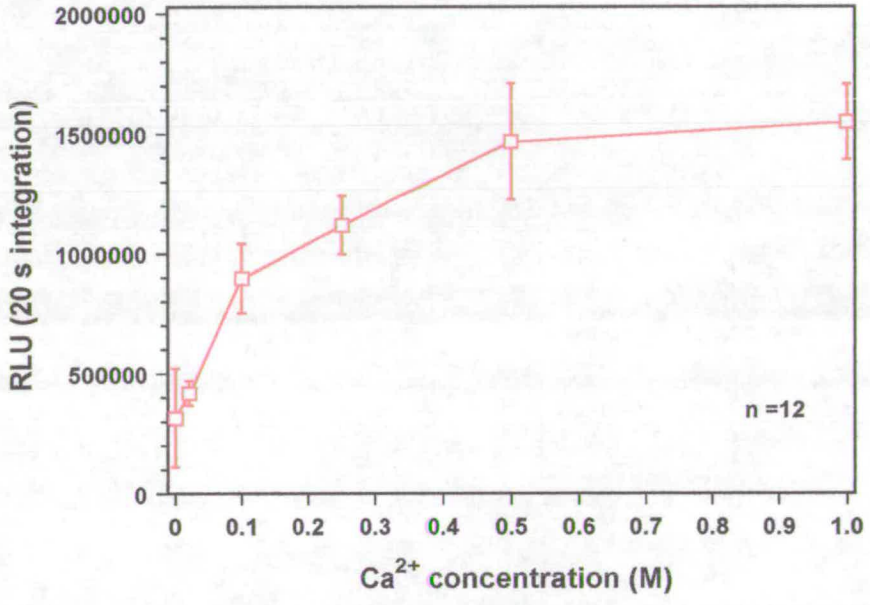
6.3.3 *Response to external Ca^{2+} stimuli*

Throughout this study, 100 mM $CaCl_2$ has been used as a standard stimulus for analysing aequorin responses both within living hyphae and *in vitro*, as the responses obtained were very large and repeatable (Figures 6.4, 6.7 and 6.9). This treatment

was analysed further to ascertain how Ca^{2+} is transported into the cytosol to elicit a response.

The effect of increased $[Ca^{2+}]$ in the media was assessed (Figure 6.10). As shown, the *in vivo* response to increasing $[Ca^{2+}]$ reaches a maximum around 500 mM $CaCl_2$ (final concentration 250 mM). Statistical analysis (using a single factor ANOVA and determining the 5% LSD) showed no significant difference above 250 mM Ca^{2+} addition, and no significant difference between 0 mM and 20 mM Ca^{2+} addition.

Figure 6.10 Mycelial response to addition of external $CaCl_2$



Key: x-axis represents concentration of $CaCl_2$ added to the wells, which diluted the media 50% (therefore final $[Ca^{2+}]$ was half that shown). y-axis represents 20 s integration of RLU after addition of solution. n = 12, error bars are ±SD.

$[Ca^{2+}]_c$ is known to be tightly regulated, and the rapid recovery of $[Ca^{2+}]_c$ back to basal level after addition of a large external $[Ca^{2+}]$ (such as shown in Figure 6.4) shows that there are very effective Ca^{2+} pumps in operation within the mycelium. In an attempt to investigate $[Ca^{2+}]_c$ regulation, several inhibitors were employed, and their effects upon the $[Ca^{2+}]_c$ response to external Ca^{2+} monitored.

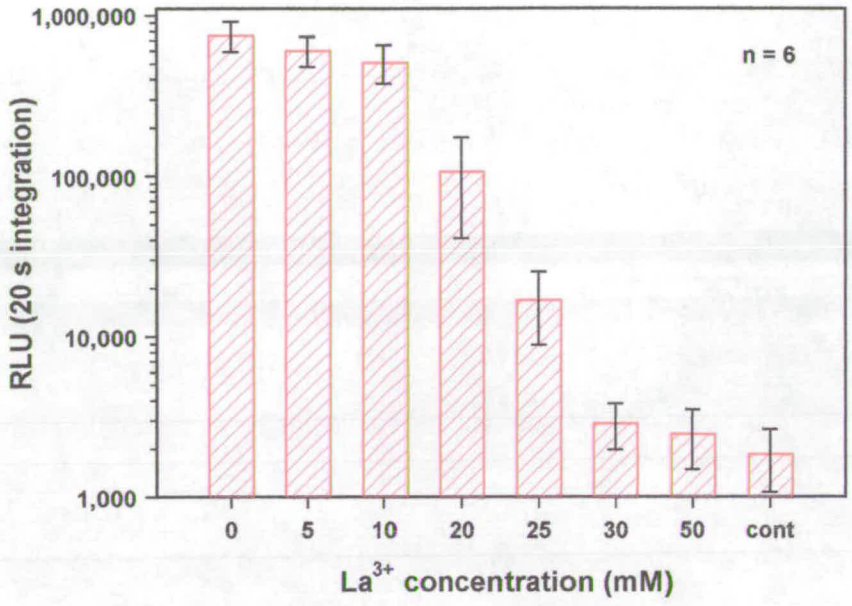
6.3.4 Inhibition of external Ca^{2+} response

To ascertain whether Ca^{2+} uptake from the media was through plasma membrane channels in the mycelia and not due to damaged plasma membrane or a very strong electrochemical gradient, external Ca^{2+} channel inhibitors were used. These act either competitively or antagonistically to inhibit Ca^{2+} flow through plasma membrane channels.

Lanthanum is a competitive inhibitor of Ca^{2+} and therefore will inhibit all plasma membrane Ca^{2+} channels (Hill *et al.*, 1998; Bellamine *et al.*, 1998). It is larger than Ca^{2+}, and when bound to a Ca^{2+} channel it blocks the channel. Since La^{3+} is too large to pass through Ca^{2+} channels, it should not interact with Ca^{2+} binding proteins within the cell, such as aequorin, which would make interpretation of the results difficult. Young cultures were pre-incubated with varying concentrations of $LaCl_3$ prior to being stimulated by the addition of external $CaCl_2$ (Figure 6.11).

Analysis of the data presented in Figure 6.11 using a single factor ANOVA and determining the 5% LSD (Figure 6.12), showed that at concentrations of La^{3+} $\geq$20 mM there was no significant difference in the response compared to the background count ('cont').

Figure 6.11: Effect of $LaCl_3$ upon external Ca^{2+} response



Key: 18 h cultures used. La^{3+} treatments added 5 min before Ca^{2+} stimulation in a total volume of 25 µl VS media. Luminescence measurements integrated for 20 s after addition of 100 mM $CaCl_2$ to each well. x-axis represents final $LaCl_3$ concentration. 'cont' represents background luminescence in 0 mM La^{3+} dataset prior to Ca^{2+} addition. n = 6, error bars are ±SD.

Figure 6.12: Tabular representation of significance of data from Figure 6.11

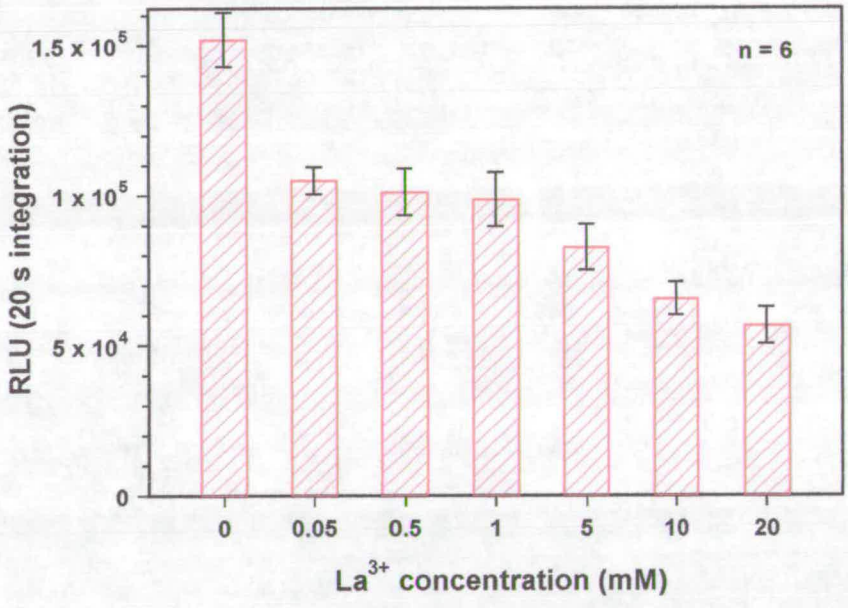
0mM	5mM	10mM	20mM	25mM	30mM	50mM	cont
752533.2	604648.8	512840.8	106805.5	16991.6	2889.0	2487.3	1853.3

Key: 5% LSD = 106347.4. Mean values shown for each treatment. Lines represent non significant difference between datasets.

As shown by the statistical analysis, La^{3+} has a sharp effect between 10 and 20 mM upon the response to addition of 100 mM $CaCl_2$. At greater concentrations, the inhibition was large, and the inhibitor concentration could be increased to a high enough concentration to almost completely quench luminescence. Repeated stimulus of the wells with addition of a further 100 mM $CaCl_2$ showed continued competition for Ca^{2+} channels in those wells containing a high enough concentration of La^{3+}, although the inhibitory effect was less due to the previous addition of Ca^{2+} to the wells increasing the overall external [Ca^{2+}] (data not shown). The concentrations

of La^{3+} required for 50% inhibition (between 10-20 mM $LaCl_3$) appear very high compared to some of those published (K0.5 = 0.11 mM La3+ in N. crassa, Corzo and Sanders, 1992; 70% inhibition of Ca2+ uptake by 0.1-1 mM La3+, Marshall *et al.*, 1994). However, Friedman *et al.* (1998) required between 10-30 mM La^{3+} to inhibit gravitropic response in *Antirrhinum majus*. The high La^{3+} concentration required for inhibition may be due to the high concentration of external Ca^{2+} added. Therefore the experiment was repeated with 10 mM $CaCl_2$ as the stimulus (Figure 6.13). As can be seen, high concentrations of La^{3+} are still required for inhibition, with 50% inhibition lying between 5-10 mM $LaCl_3$. To determine why the IC_{50} for La^{3+} inhibition in *Aspergillus* differs from other species requires further work.

Figure 6.13: Effect of $LaCl_3$ upon response to 10 mM $CaCl_2$ addition

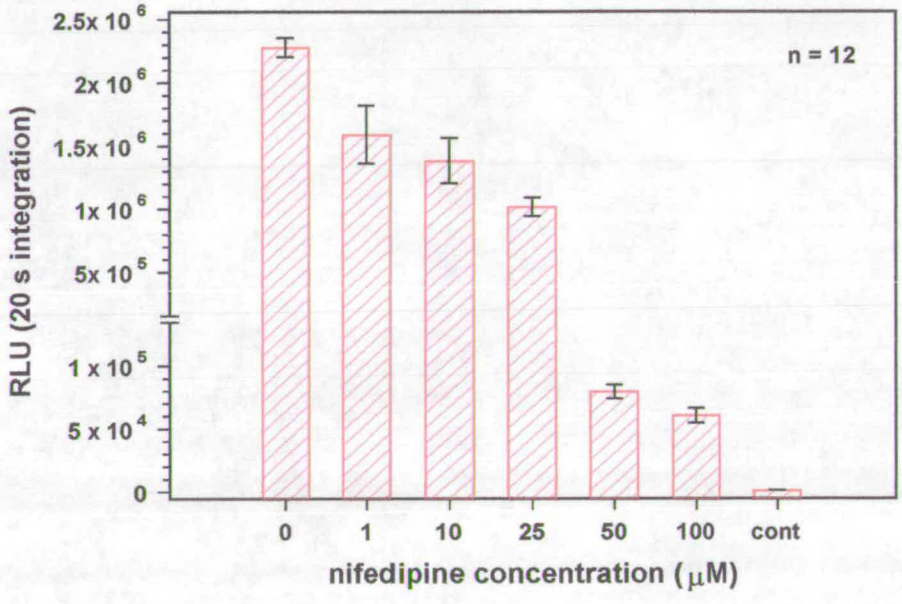


Key: 18 h cultures used. La^{3+} treatments added 5 min before Ca^{2+} stimulation in a total volume of 25 µl VS media. Luminescence measurements integrated for 20 s after addition of 10 mM $CaCl_2$ to each well. x-axis represents final $LaCl_3$ concentration. n = 6, error bars are ±SD.

Nifedipine acts as an antagonist, blocking L-type Ca^{2+} channels (Ishihara *et al.*, 1996; Kondo *et al.*, 1995). Treatment with nifedipine was carried out as described

for La^{3+}. Nifedipine was seen to inhibit aequorin luminescence in a dose-dependent manner, showing that Ca^{2+} uptake occurs through nifedipine-sensitive Ca^{2+} channels (Figure 6.14). Continuing to increase the nifedipine concentration up to 100 μM showed an almost complete inhibition (97.3%) of luminescence, which suggests that almost all Ca^{2+} uptake is through nifedipine-sensitive Ca^{2+} channels.

Figure 6.14: Effect of nifedipine upon Ca^{2+} uptake in mycelia



Key: nifedipine dissolved in a minimal volume of MeOH (≤ 1 μl per well). Treatments added 5 min before Ca^{2+} stimulation in a total volume of 25 μl VS media. Control wells pre-incubated with 25 μl VS + MeOH (1 μl). Measurements taken for 20 s after addition of 100 mM $CaCl_2$ to the well. x-axis represents final nifedipine concentration. 'cont' represents 20 s background luminescence count before stimulation in the 0 μM nifedipine wells. n = 12, error bars are ±SD

Figure 6.15: Tabular representation of significance of data from Figure 6.14

0 μM	1 μM	10 μM	25 μM	50 μM	100 μM	cont
2277641	1585546	1381119	1015309	79284.2	60415.3	877.7

Key: 5%LSD=332390.4. Means for each treatment shown. Underlined points represent no significant difference between the datasets.

Analysis of the data using a single factor ANOVA and determining the 5% LSD showed that there was no significant difference between the 'cont' background count, 100 μM and 50 μM nifedipine. Also, there was no significant difference between 10 μM and 1 μM nifedipine. All other concentrations were significantly different from one another (Figure 6.15).

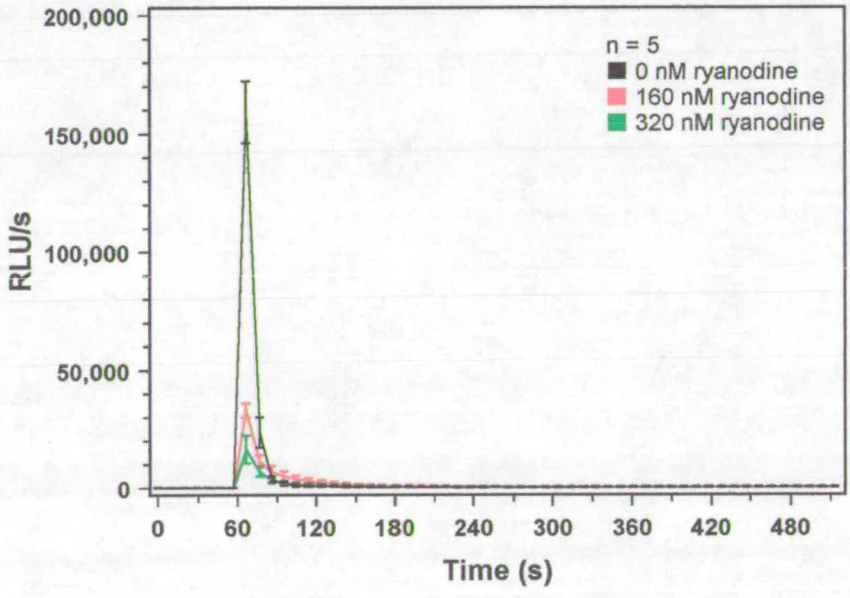
Other external Ca^{2+} channel inhibitors were tried, such as gadolinium, which acts in a similar way to La^{3+}, and is also an inhibitor of stretch-activated channels (Hill *et al.*, 1998; Takenaka *et al.*, 1998; Rock and Quatrano, 1996). Similar results were obtained (data not shown), although it was more difficult to interpret data from the gadolinium series of experiments, as it formed a precipitate of gadolinium phosphate when added to the media (all media used contained phosphate). This turned the media opaque, which will have limited the amount of detectable luminescence, and also sequestered some of the gadolinium into precipitate, so that the final concentration was unknown.

It has been hypothesised that there exists a positive feedback loop to help amplify the Ca^{2+} response to an external signal (Berridge, 1993a). This operates by the increase in $[Ca^{2+}]_c$ from external Ca^{2+} sources causing release of Ca^{2+} from internal stores, and is known as the Calcium Induced Calcium Release (CICR) theory. Use of internal Ca^{2+} channel antagonists in the presence of a known external stimulus should help show whether this occurs in this system. Ryanodine and TMB-8 (3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester; Calbiochem, UK) are two such inhibitors. Ryanodine is known to block the release of Ca^{2+} from the sarcoplasmic reticulum (SR) in mammalian cells (McPherson and Campbell, 1993b). TMB-8 is

an intracellular Ca^{2+} antagonist that blocks the release of Ca^{2+} from intracellular stores (Eidne *et al.*, 1994; Warburton and Deacon, 1998).

Incubation with ryanodine prior to stimulation of mycelia with external Ca^{2+} showed a large decrease in luminescence which was dose-dependent (Figure 6.16).

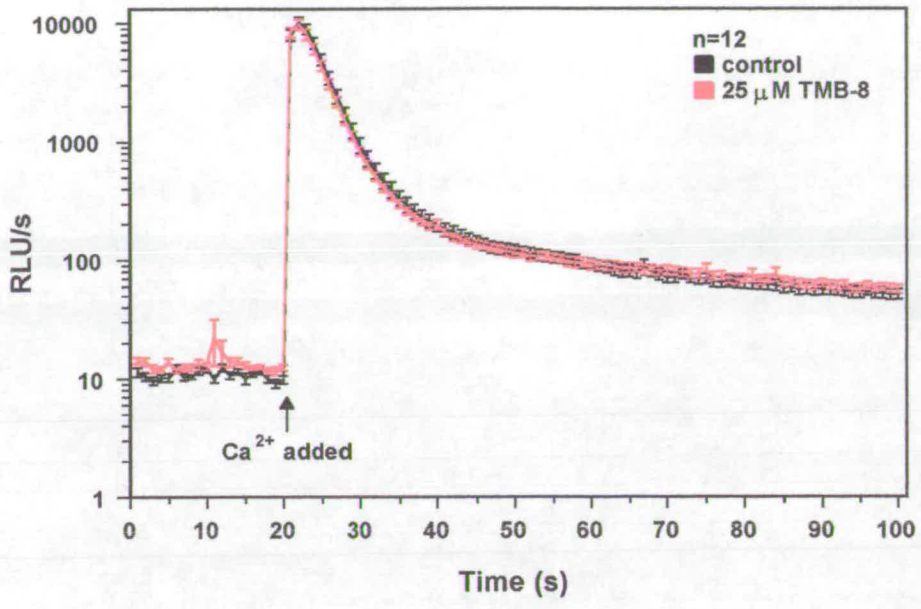
Figure 6.16: Effect of ryanodine on mycelial $[Ca^{2+}]_c$ response to external Ca^{2+}



Key: ryanodine dissolved in a minimal volume of acetone (final acetone concentration ≤ 0.8% v/v per well) and added to the wells in 25 µl VS. Control wells pre-incubated with 25 µl VS + acetone (final concentration 0.8% v/v). Pre-incubations carried out 5 min prior to start of luminescence measurements. Final ryanodine concentrations shown in figure. 100 mM $CaCl_2$ added at $t = 60$ s. n = 5, error bars are ±SD.

Incubation of mycelia with TMB-8 prior to stimulation with external $CaCl_2$ showed no difference in the mycelial response (Figure 6.17). The concentration of TMB-8 used was based on evidence shown in Section 6.5.1, where the effects of TMB-8 are discussed in more detail.

Figure 6.17: Effect of TMB-8 on $[Ca^{2+}]_c$ response to external $CaCl_2$



Key: 18 h cultures used. 25 µl of either VS or VS + 125 µM TMB-8 added to wells 5 min prior to measurement. 100 mM $CaCl_2$ added at t = 20 s. n = 12, error bars are ±SD.

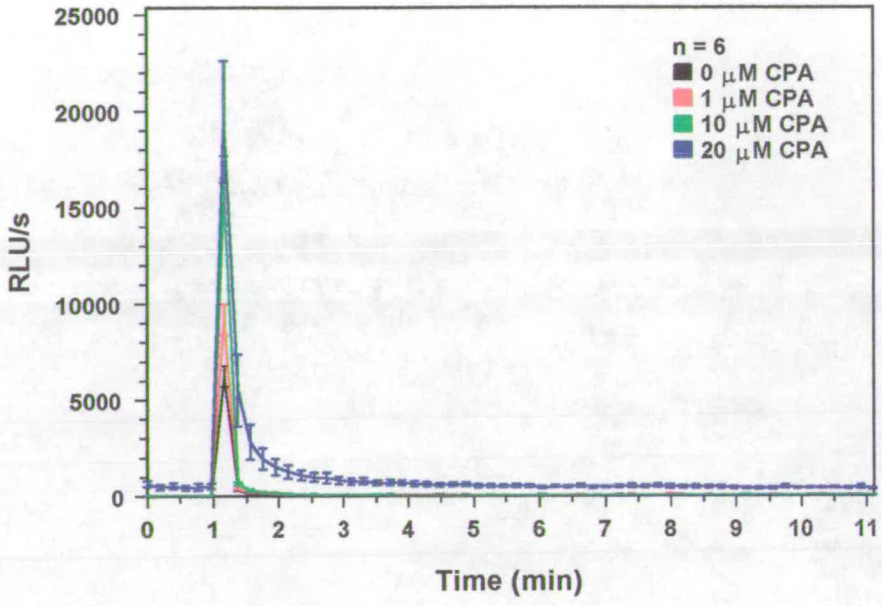
It is difficult to draw a conclusion from these results, as it might be expected that if CICR occurs upon stimulation with external Ca^{2+}, then the $[Ca^{2+}]_c$ increase would be inhibited in the presence of TMB-8 as well as in the presence of ryanodine. It is possible that ryanodine is acting upon some other part of the Ca^{2+} signalling network, or indeed upon aequorin itself. The concentrations of ryanodine used are quite high compare to those published (40 nM causes 50% inhibition in red beet vacuolar microsomes Muir and Sanders, 1996). Also, TMB-8 increases the basal luminescence level over time, which ryanodine does not (data not shown). There are slight differences in the action of the two inhibitors; ryanodine blocks ryanodine receptors which are involved in cADP ribose-induced Ca^{2+} release, and in plant vacuoles TMB-8 shows selectivity for blocking IP_3-induced Ca^{2+} release (Allen and Sanders, 1997) which ryanodine is ineffective against (Muir *et al.*, 1997). Therefore, the $[Ca^{2+}]_c$ increase observed is possibly enhanced by cADP ribose-induced Ca^{2+} release in response to external Ca^{2+}.

Further work was carried out to try and determine where the influx of Ca^{2+} was rapidly removed to. The options available are to pump cytosolic Ca^{2+} either into an internal store such as the ER or vacuole, or to pump Ca^{2+} out of the cell into the media. Ca^{2+}-ATPase pumps are required for this, to move Ca^{2+} up the electrochemical gradient. There are known inhibitors for these channels, which, if used in the presence of a large influx of Ca^{2+} into the cytosol should help determine which channels are used to regulate $[Ca^{2+}]_c$.

Cyclopiazonic acid (CPA, Calbiochem) is a reversible inhibitor of ER Ca^{2+}-ATPase, and therefore blocks uptake of Ca^{2+} from the cytosol by these channels (Schaefer *et al.*, 1994). Incubation of young mycelial cultures with CPA prior to stimulation with Ca^{2+} showed an increase in luminescence directly upon stimulation, but no large effect upon recovery from the stimulus (Figure 6.18). A slower recovery rate (as shown by the increased luminescence) was only observed in the presence of 20 μM CPA.

Statistical analysis of the peaks shown in Figure 6.18 in response to addition of $CaCl_2$ shows there is a significant increase in response to addition of Ca^{2+} in the presence of ≥ 10 μM CPA (Figure 6.19), which suggests that regulation occurs very rapidly after addition of external Ca^{2+} and that CPA-sensitive Ca^{2+}-ATPase channels are used for regulation. However, since there is little effect upon recovery, stores other than the ER are used for long term $[Ca^{2+}]_c$ regulation.

Figure 6.18: Effect of cyclopiazonic acid on mycelial $[Ca^{2+}]_c$ response to external Ca^{2+}



Key: 18 h cultures used. CPA dissolved in $CHCl_3$ to a concentration of 20 mM. Dilutions made in VS medium and added 5 min prior to measurement in 25 µl VS (final $CHCl_3$ concentration ≤ 0.005% v/v). 0 µM CPA wells incubated with 25 µl VS + $CHCl_3$ (0.005% v/v) Final CPA concentrations shown in figure. 100 mM $CaCl_2$ added at t = 1 min. n = 12, error bars are ±SD.

Figure 6.19: Tabular representation of significance of data shown in Figure 6.18

0 µM CPA	1 µM CPA	10 µM CPA	20 µM CPA
6217.83	9175.00	15899.67	19458.33

Key: 5% LSD = 5545.56. Mean values shown from peak response after addition of $CaCl_2$ to wells. Lines represent non significant difference between datasets.

6.3.5 Conclusion

The work presented in this Section shows that uptake of Ca^{2+} occurs through specific plasma membrane Ca^{2+} channels. The investigation of the possibility of CICR occurring with an external Ca^{2+} stimulus was inconclusive, although ryanodine sensitive Ca^{2+} channels appear to be involved in releasing Ca^{2+} from internal stores.

If this is the case, then the channels involved are not sensitive to TMB-8. $[Ca^{2+}]_c$ regulation appears very efficient, and the data obtained with CPA suggests that Ca^{2+}-ATPase pumps are involved in removal of Ca^{2+} from the cytosol.

6.4 Response to touch and osmotic shock

The previous section has outlined the success of using synthetic aequorin to measure large changes in $[Ca^{2+}]_c$. However, measurement of mycelial response to more physiologically acceptable stimuli is of more interest. As shown earlier, it is possible to monitor the basal level of $[Ca^{2+}]_c$ in cultures of growing hyphae from a few hours after inoculation (Figure 6.5). As mycelial density increases, so too does the amount of aequorin present, therefore raising the background level of luminescence, up until sporulation inhibits the passage of light from the well to the photo-multiplier tube (Figure 6.6). Therefore, using cultures aged between 18 and 36 h, small changes in luminescence can be monitored. Such cultures were used to monitor the effects of media addition.

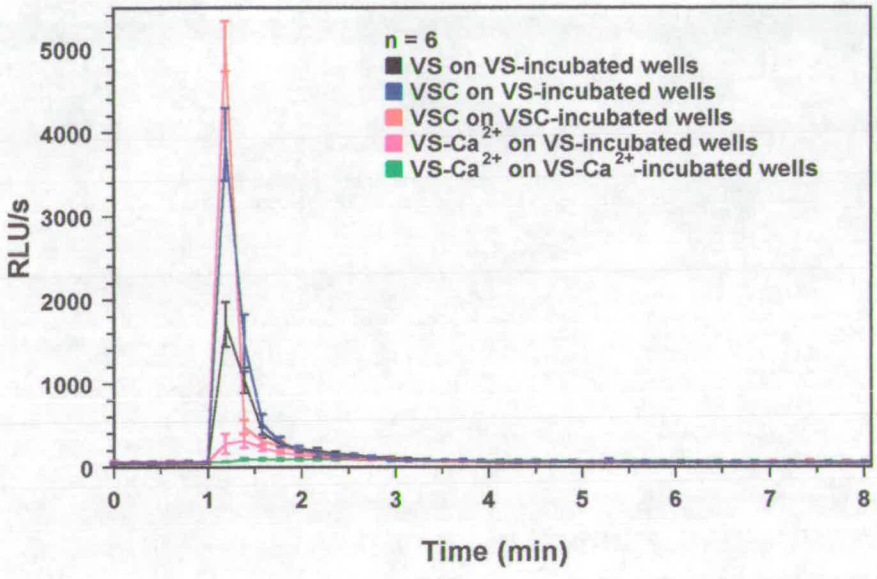
6.4.1 Addition of iso-osmotic media

A typical response to the addition of an equal volume of iso-osmotic media is shown in Figure 6.20. The response is very small, but lasts for a relatively long time compared to the response to external Ca^{2+}. It takes about 8 min for the luminescence to reach resting level after stimulation. Since the added media is iso-osmotic to the media the cultures are already in, the response appears to be one of a mechanical stimulus.

This response can be shown to rely upon external Ca^{2+} by adding modified medium to the cultures (Figure 6.20). Addition of medium with added Ca^{2+} (VSC, $[Ca^{2+}] = 3$ mM) increases the luminescence response both when added to cells in

normal medium and cells in VSC medium. Conversely, addition of medium with no Ca^{2+} (VS-Ca^{2+}, Vogel's without $CaCl_2$) reduces the iso-osmotic response. For all media, the magnitude of the response relies upon the total external [Ca^{2+}].

Figure 6.20: Mycelial response to addition of media with different Ca^{2+} concentrations



Key: media removed from 24 h cultures and replaced with 100 µl of either normal VS, VS + 3 mM $CaCl_2$ (VSC) or VS without Ca^{2+} (VS-Ca^{2+}). Cultures left for 15 min to recover, then luminometry carried out. 100 µl of the appropriate medium added at t = 1 min. n = 6, error bars are ±SD.

The final external Ca^{2+} concentrations for each experiment shown in Figure 6.20 are as shown in Table 6.3, assuming that the [Ca^{2+}] in VS-Ca^{2+} is 0 µM.

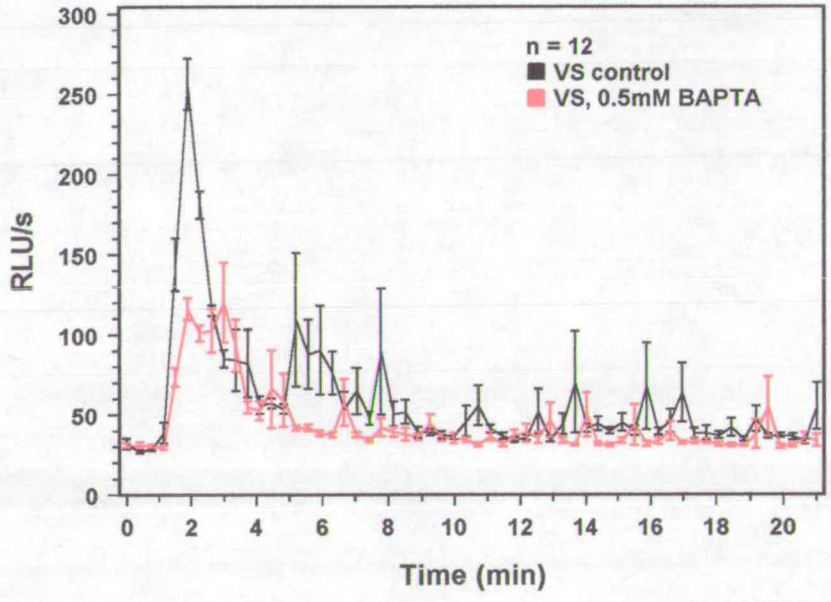
Table 6.3: Final external Ca^{2+} concentrations for experiments shown in Figure 6.20

experiment:	VS on VS	VSC on VS	VSC on VSC	VS-Ca^{2+} on VS	VS-Ca^{2+} on VS-Ca^{2+}
[Ca^{2+}] (mM):	0.68	1.84	3	0.34	0

Several inhibitors and modulators were used to characterise the iso-osmotic response. BAPTA (BAPTA tetrasodium salt, [1,2-*bis*(*o*-aminophenoxy)ethane-

N,N,N',N'-tetraacetic acid, sodium], Sigma) is a membrane impermeant Ca^{2+} chelator which lowers the external $[Ca^{2+}]$ in the same manner as EGTA (Section 6.3.1). Addition of media in the presence of BAPTA shows a decrease in response, confirming that at least part of the $[Ca^{2+}]_c$ increase comes from external Ca^{2+} stores (Figure 6.21).

Figure 6.21: Mycelial response to addition of media and the effect of exogenous BAPTA

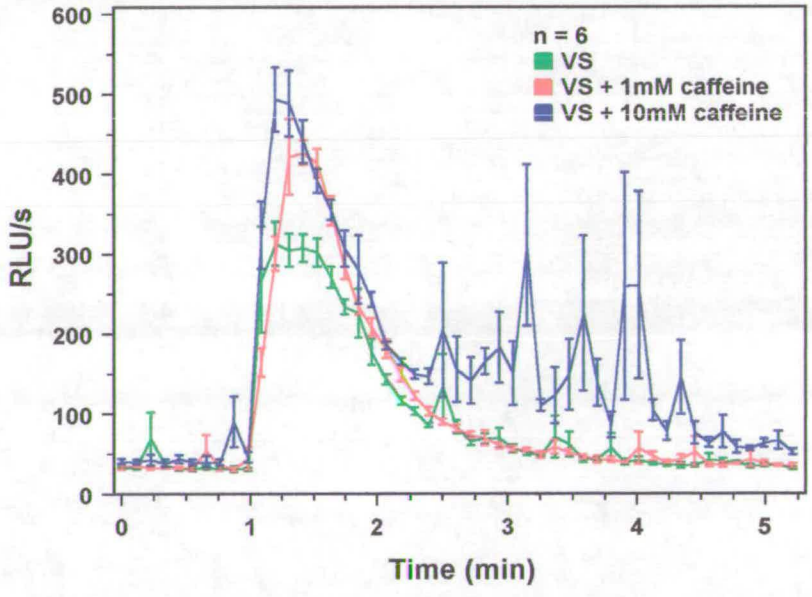


Key: 18 h cultures used. Wells pre-incubated for 5 min with either 25 µl VS medium (control) or 25 µl VS + 2.5 mM BAPTA (final concentration 0.5 mM). 100 µl VS medium added at t = 1.25 min. n = 12, error bars are ±SD.

Caffeine activates the intracellular ryanodine receptors which release Ca^{2+} from internal stores, and can be shown to have an effect upon *A. awamori* mycelia, as shown by its effect upon the mechanical stimulus (Figure 6.22). Pre-incubation with caffeine causes an apparently slightly higher basal luminescence level. However, the difference seen is too slight to be significant. Upon stimulation with media, the caffeine-incubated mycelia show a higher level of luminescence than the control cultures. Also, at higher concentrations (10 mM), caffeine is seen to prolong the high $[Ca^{2+}]_c$ (Figure 6.22). It is difficult to interpret these results with respect to the

media response, as it does not conclusively prove that ryanodine receptors are stimulated by this response as caffeine is an agonist. However, it does prove that ryanodine receptors are present in *A. awamori*. Using ryanodine, which is an antagonist for ryanodine receptors should show whether the release of Ca^{2+} is stimulated from ryanodine receptor-mediated Ca^{2+} stores. However, use of ryanodine up to a final concentration of 240 nM shows no difference in response to media addition (data not shown), which suggests that ryanodine receptors are not stimulated by response to media.

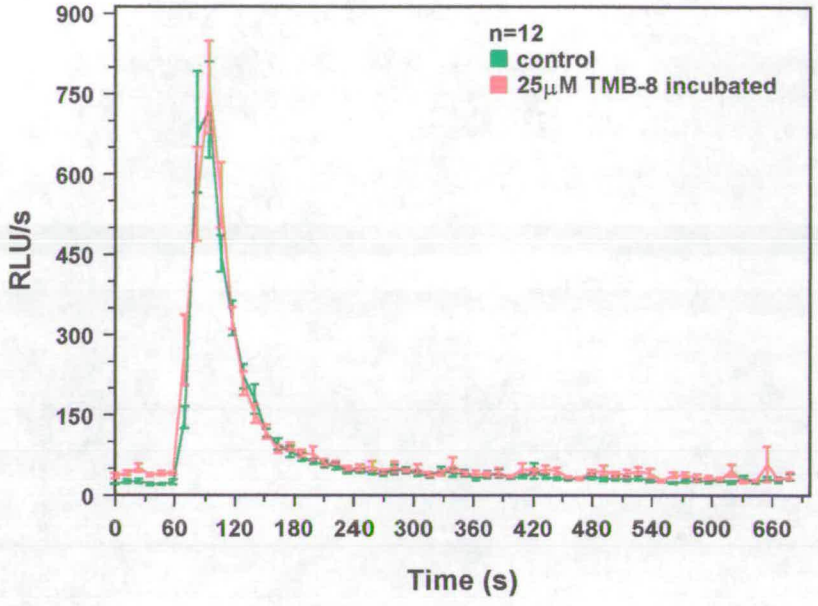
Figure 6.22: Stimulation of ryanodine receptors by caffeine during response to media addition



Key: 18 h cultures used. Wells pre-incubated with 25 µl VS medium (+ caffeine where required) for 5 min. Iso-osmotic VS media added at t = 1 min. Each treatment is significantly different when luminescence is integrated over the period 1-2 min (analysis of variance gave a p value < 0.05). n = 6, error bars are ±SD.

As described for the response to external Ca^{2+}, TMB-8 was also used to determine whether intracellular Ca^{2+} stores are used in the response to media addition. As shown in Figure 6.23, there is no difference in response to media in the presence or absence of TMB-8. This suggests that internal Ca^{2+} stores are not used in the media response, which agrees with the data obtained for ryanodine in this instance.

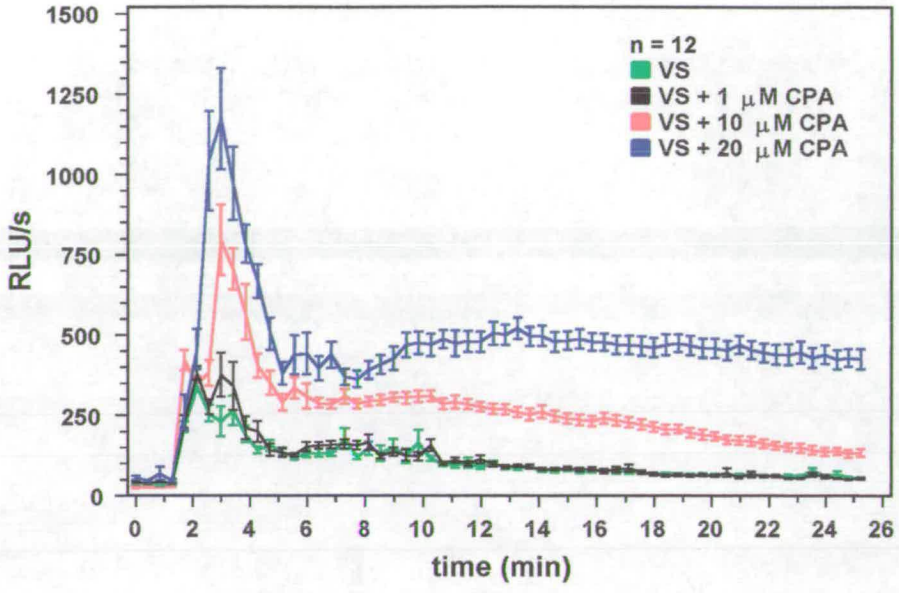
Figure 6.23: Effect of TMB-8 upon response to addition of media



Key: 18 h cultures used. Wells pre-incubated for 5 min with either 25 μl VS (control) or 25 μl VS + 125 μM TMB-8 prior to luminometry. Iso-osmotic media added at t = 60 s. n = 12, error bars are ±SD.

To provide evidence for whether ER Ca^{2+}-ATPases are involved in the recovery of the $[Ca^{2+}]_c$ increase after stimulation by the addition of media, cyclopiazonic acid was employed. As shown in Figure 6.24, the mechanical response takes longer to return to basal level in the presence of CPA, with the initial response also being larger in the presence of CPA, which suggests that regulation of $[Ca^{2+}]_c$ begins almost immediately after stimulation. This is the same result as that obtained with an external Ca^{2+} stimulus (Figure 6.18). However, in this case there is marked inhibition of $[Ca^{2+}]_c$ recovery in the presence of 10 μM and 20 μM CPA.

Figure 6.24: Effect of cyclopiazonic acid upon response to media addition



Key: CPA dissolved in $CHCl_3$. Final $[CHCl_3] \leq 0.005\%$v/v. wells pre-incubated for 5 min with either 25 µl VS + $CHCl_3$ (0.005% v/v) or 25 µl VS + CPA (at final concentrations shown) prior to luminometry. Iso-osmotic media added at $t = 1.25$ min. n = 12, error bars are ±SD.

It is difficult to determine from the frequency of the datapoints taken, but the response appears to be bimodal, with a second increase in $[Ca^{2+}]_c$ occurring approx. 2 min after stimulation ($t = 3.25$ min). This is most noticeable with 1 µM CPA, and to a lesser extent with 10 µM CPA. With a higher concentration of CPA (20 µM) the second response is so large that it blurs the modality of the response to media addition. Statistical analysis of the initial response and the secondary response shows that there is no difference in the initial response between treatments (Figure 6.25). Both 10 µM and 20 µM CPA treatments produce a significant increase in luminescence for the second response. Using a two factor ANOVA, a 5% LSD of 348.95 was calculated, which if compared to the means shown in Figure 6.25 shows that there is only a significant increase in between the primary and secondary responses in the presence of 10 µM and 20 µM CPA. It can be seen that luminescence in the control wells decreases between the primary and secondary responses (i.e. no secondary response is seen), but the difference is not statistically significant compared to the increases seen with high concentrations of CPA.

Figure 6.25: Tabular representation of significance of data from Figure 6.24

Initial response (x = 1.75 + 2.15 min from Figure 6.24)

0 μM CPA	1 μM CPA	20 μM CPA	10 μM CPA
556.42	610.92	717	772.92

Second response (x = 3 + 3.4 min from Figure 6.24)

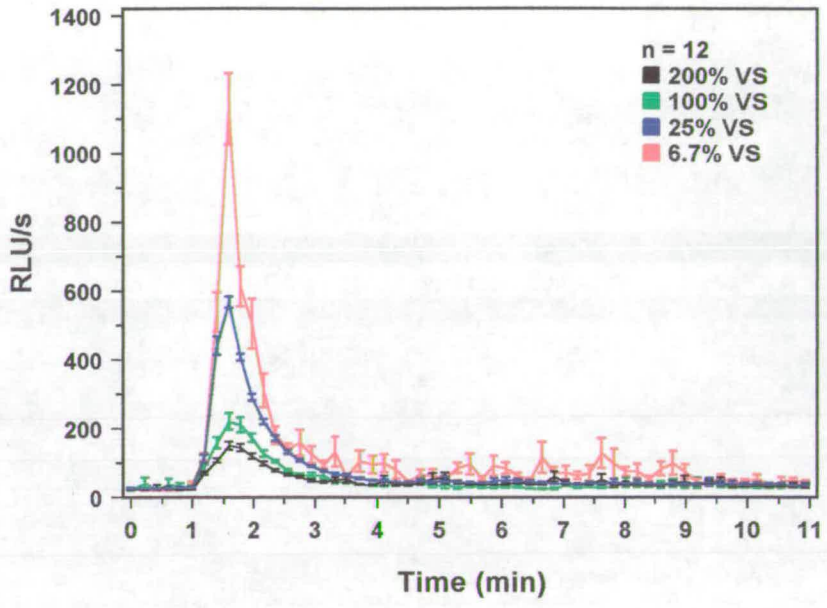
0 μM CPA	1 μM CPA	10 μM CPA	20 μM CPA
497.67	717.83	1502.83	2145.67

Key: Mean total RLU/s taken from both time measurements indicated for each response from Figure 6.24. A two factor ANOVA determined a 5% LSD of 348.95 between the two responses. Underlined datasets represent non significant differences for the 5% LSD.

6.4.2 Addition of hypo-osmotic media

Previous studies using aequorin as a reporter have shown a response to hypo-osmotic shock in *S. cerevisiae* (Batiza *et al.*, 1996). Using this work as a template, mycelial cultures were treated with VS medium and dilutions thereof. As can be seen in Figure 6.26, the greater the hypo-osmotic shock, the greater the response obtained.

Figure 6.26: Mycelial response to hypo-osmotic shock



Key: 18 h cultures used. 100 µl cultures injected at 1 min 9 s with 100 µl of the appropriate medium. n = 12, error bars are ±SD.

Analysis of the data from stimulation up until the peak of the response shows each treatment to be significantly different from each other, except for normal media (100% VS) and hyper-osmotic media (200% VS) (Figure 6.27).

Figure 6.27: Statistical analysis of response to media addition

200% VS	100% VS	25% VS	6.7% VS
401.00	590.17	1414.09	2279.75

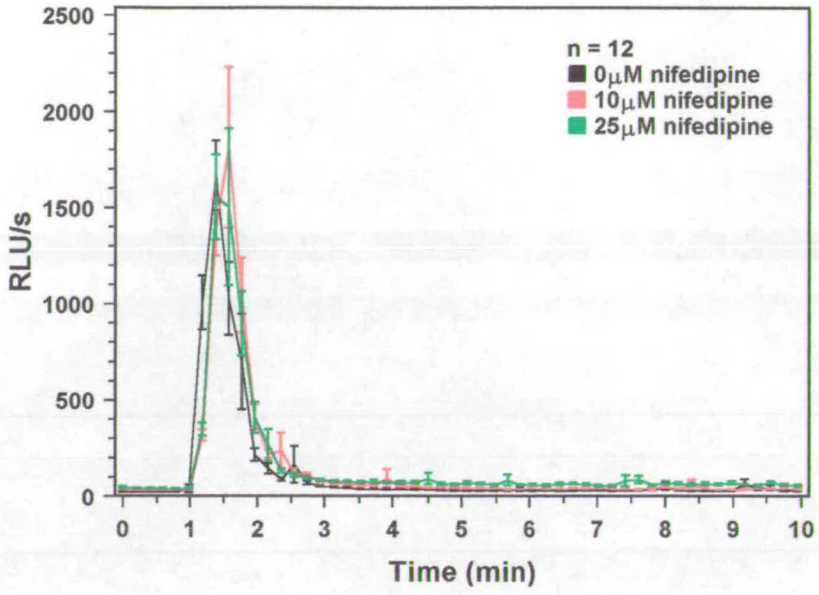
Key: Mean RLU measurements shown, representing the mean total RLU from t = 1 min 23 s to 1 min 46 s for each treatment (Figure 6.26). 5% LSD = 279.83. Underlined datasets represent non significant differences for the 5% LSD.

Although the initial response increases with the increase in hypo-osmotic shock, the time to recover back to basal luminescence remains the same. This suggests that the mycelia is able to control $[Ca^{2+}]_c$ in response to these treatments. We can hypothesise that the response to hypo-osmotic shock is made up of the mechanical stimulus response seen when adding iso-osmotic media and a further response to the

dilution of the medium. Treatment of mycelial cultures with hyper-osmotic media does not increase the luminescence response. This correlates with the available evidence for osmosensing. In yeast, two separate pathways appear to be used for hyper-osmotic and hypo-osmotic shock. Yeast cells respond to hyper-osmotic shock by activating a mitogen-activated protein (MAP) kinase cascade, which is called the high osmolarity glycerol (HOG) response pathway (Maeda *et al.*, 1994). In comparison, hypo-osmotic shock causes an increase in $[Ca^{2+}]_c$ (Batiza *et al.*, 1996) and has been shown to activate the protein kinase C1 MAP kinase pathway (Davenport *et al.*, 1995). This has also been shown in rat nerve cells (Mongin *et al.*, 1997), and to be a pre-requisite for activation of downstream protein kinases in tobacco cells in response to hypo-osmotic shock (Takahashi *et al.*, 1997a; Takahashi *et al.*, 1997b)

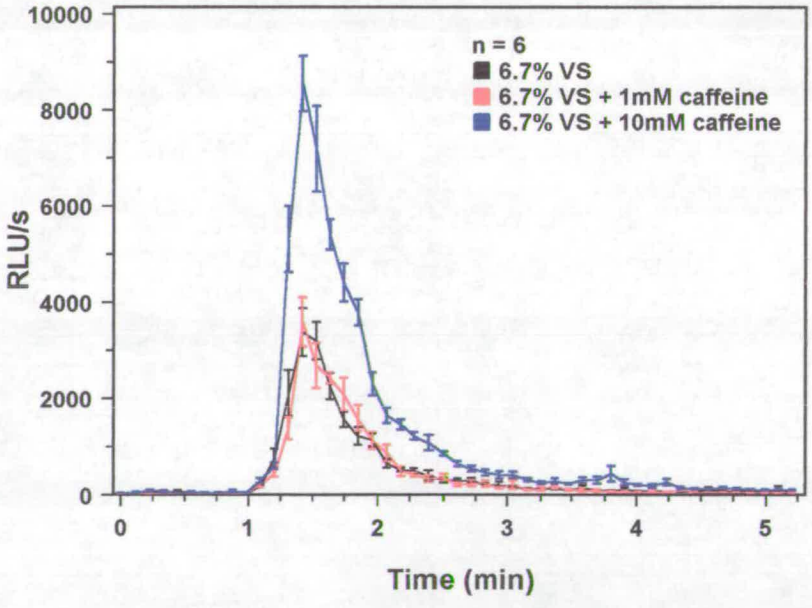
The response to hypo-osmotic shock was investigated using the same Ca^{2+} modulators as those described for characterising the media response. Interpretation of the data is made difficult by the fact that we can expect to see the responses to mechanical stimulus as well as any response to the hypo-osmotic shock. As shown in Figure 6.28, the use of BAPTA produces the same result as obtained with addition of iso-osmotic media (Figure 6.21).

Figure 6.29: Effect of nifedipine upon response to hypo-osmotic shock



Key: wells pre-incubated for 5 min with 25 μl VS + MeOH or 25 μl + nifedipine (dissolved in MeOH). Final MeOH concentration not exceeding 1% v/v. 6.7% VS medium added at $t = 1$ min. n = 12, error bars are ±SD.

Figure 6.30: Effect of caffeine upon mycelial response to hypo-osmotic shock



Key: wells pre-incubated with 25 μl VS medium (+ caffeine where required) for 5 min. 6.7% VS media added at $t = 1$ min. n = 6, error bars are ±SD.

Figure 6.31: Tabular representation of statistical significance from Figure 6.30

1 mM caffeine	0 mM caffeine	10 mM caffeine
14427.17	14666.33	35560.67

Key: Figures show mean total RLU from t = 1 min to 2 min from Figure 6.30. 5% LSD = 5769.57. Line represents non significantly different datasets.

The inhibitors TMB-8 and CPA were both used to analyse mycelial response to hypo-osmotic shock. The results obtained were no different from those shown for response to iso-osmotic media (Section 6.4.1).

6.4.3 Conclusion

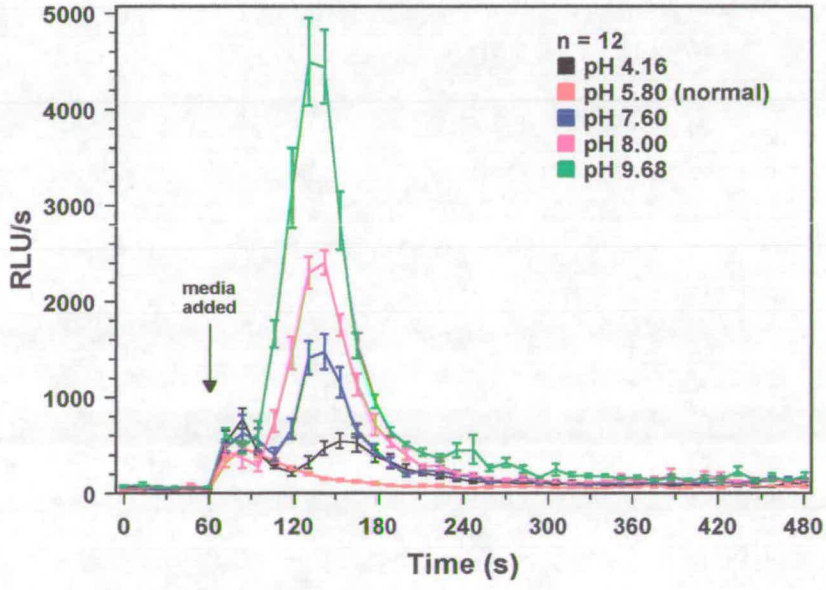
Overall, data from the experiments carried out suggest that there is no distinguishable difference in response to iso-osmotic and hypo-osmotic media except that the magnitude of the $[Ca^{2+}]_c$ increase is greater in response to hypo-osmotic stimulation. Both $[Ca^{2+}]_c$ responses seem to use the same method for Ca^{2+} uptake and recovery. External Ca^{2+} is required for the media response. TMB-8 sensitive Ca^{2+} channels are not stimulated and therefore intracellular stores are apparently not used, although TMB-8 may be specific only to IP_3-sensitive stores. However, use of CPA indicates that ER Ca^{2+}-ATPases are used for recovery from the response. The results obtained for the hypo-osmotic response fit with the published observations for the same stimulus in yeast (Batiza *et al.*, 1996).

6.5 Response to external pH

Since response to media addition has been shown and characterised to a certain extent, altering the pH of the medium was investigated to ascertain whether modification of external pH affected $[Ca^{2+}]_c$.

The medium used throughout this study (VS) has a normal pH of 5.80. The pH of media was adjusted using either NaOH or HCl. As shown in Figure 6.32, when the pH of the added media was adjusted there was a secondary response 2-3 min after addition of the medium. The greater the difference in the pH of the added media, the greater the secondary response. The normal, 'primary' mechanical response to iso-osmotic medium addition still occurs (Figure 6.20 and Figure 6.32)

Figure 6.32: Mycelial $[Ca^{2+}]_c$ response to addition of pH-modified media



Key: pH-modified media added at t = 60 s. n = 12, error bars are ±SD.

Statistical analysis of the secondary response (from t = 120 - 180 s) using a single factor ANOVA and determining the 5% LSD showed that the response is not significantly different between pH values 4.16, 5.01, 5.8 (control), 5.95 and 7.0 (Figure 6.33). However, the greater response with a larger difference in pH produces a statistically significant secondary response.

Comparison of the background luminescence in each well (0-60 s) and the secondary response (120-180 s) using a 2 factor ANOVA produces a low residual mean square,

indicating there is no interaction between the initial background count in the different datasets and the pH response.

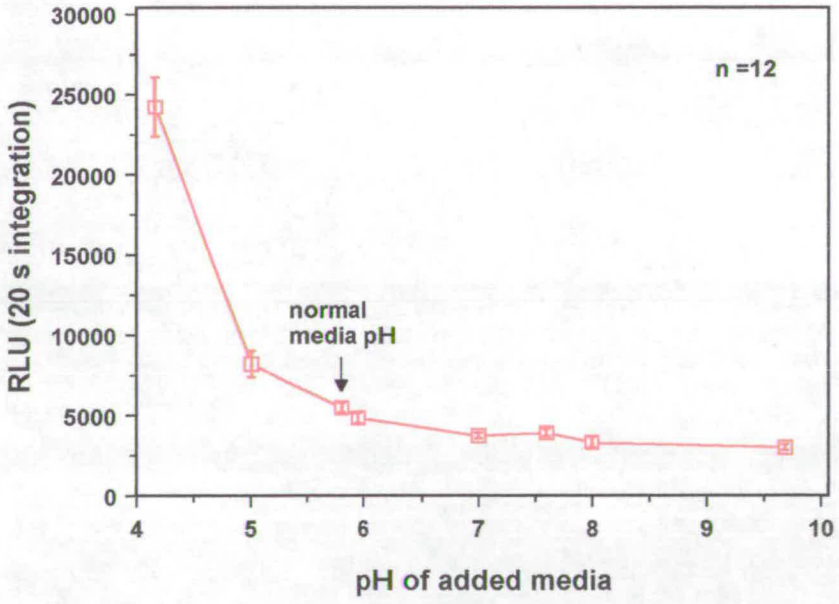
Figure 6.33: Tabular representation of statistical significance of secondary response in Figure 6.32 (t= 120-180 s)

pH	9.68	8	7.6	4.16	7.0	5.01	5.95	5.8
mean	17468.6	9775.8	5817.3	2465.7	2069.5	1183.5	1094.6	987.6

Key: mean total RLU from t = 120-180 s from Figure 6.32 shown for each treatment. 5% LSD for mean totals = 1568.5. Line represents datasets that are not significantly different from one another.

The primary response was analysed further, and shown to be higher with the addition of media more acidic than the current medium, and lower with more alkaline media over the first 20 s of response (Figure 6.34). However, for the primary response only the lower pH values (pH 4.16 and 5.01) are significantly different.

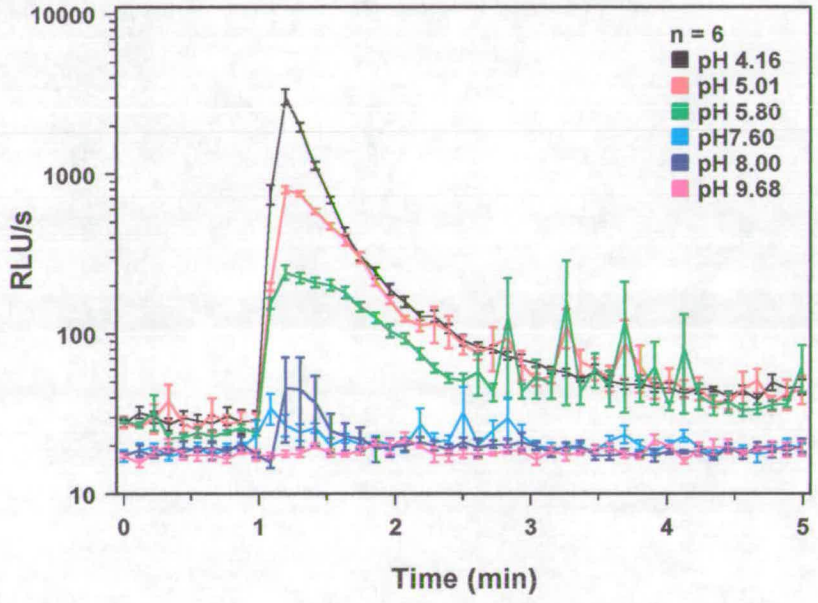
Figure 6.34: Primary response to addition of pH-modified media



Key: 30 h cultures used. Media added and luminescence monitored for 20 s. n = 12, error bars are ±SD.

Cultures grown in pH-modified media showed a normal media response upon addition of iso-osmotic medium of the same pH (iso-basic, Figure 6.35). Growth was severally inhibited at pH ≥7.6, and therefore the $[Ca^{2+}]_c$ response to iso-osmotic media addition is very slight. At pH 9.68 there was no growth observed at all, and consequently no response is observed. The more acidic the media, the greater the response to addition of media. This was not investigated further. Since there is no secondary response, such a response must be due to the difference in the pH of the added media compared to the medium that the cultures are grown in.

Figure 6.35: Response of pH-modified grown cultures to addition of iso-basic media



Key: cultures grown for 24 h in the respective pH-modified media. Iso-osmotic medium of the same pH (iso-basic) was added at $t = 1$ min. n = 6, error bars are ±SD.

6.5.1 Modulation of the pH response

Inhibitors were employed as previously to attempt to determine where the Ca^{2+} originated from for the secondary response. Use of nifedipine showed no significant difference in the secondary response, and a normal response on the primary

mechanical stimulus (data not shown). This suggested that the Ca^{2+} required for the secondary response was not obtained through L-type plasma membrane Ca^{2+} channels.

To determine whether external Ca^{2+} is required for part of the secondary response, BAPTA was used to chelate external Ca^{2+}. BAPTA is insensitive to pH, and therefore should not be affected by the addition of pH-modified media. The secondary response was analysed for the effects of incubation of cultures with BAPTA (at 0.5 mM) prior to addition of pH-modified media (Table 6.4). With the exception of pH 5.01, there is no significant difference in the secondary response (Table 6.4).

Table 6.4: Effect of BAPTA on secondary $[Ca^{2+}]_c$ response to pH-modified media

	pH	4.16	5.01	5.8	7.6	8.0	9.68
control		1355.67	1212.17	864.67	990	1100.5	1605.67
0.5 mM BAPTA		1250.83	989.5	846.67	834.17	1015.83	1403.17
		nsd	sd	nsd	nsd	nsd	nsd

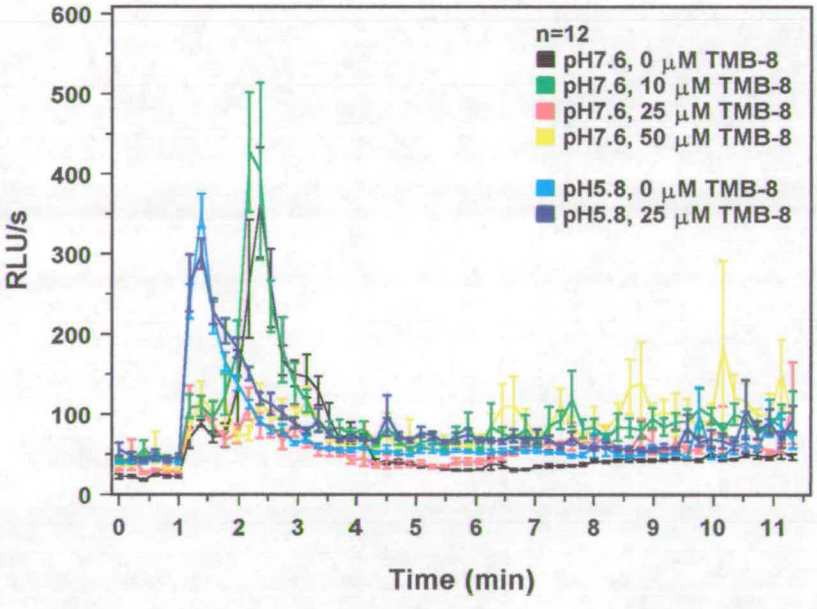
Key: 24 h cultures used. Wells incubated for 10 min with either 25 µl VS or 25 µl VS + BAPTA (final concentration 0.5 mM) prior to media addition. Values shown are RLU integrated from t = 120-180 s after media addition. 5% LSD calculated using 2 factor ANOVA with replication. 5% LSD for secondary response = 215.74. For each response, no significant difference between control and BAPTA treated wells designated as 'nsd'; significant difference between control and BAPTA treated wells designated as 'sd'. n = 6.

A shown in Table 6.4, there is a significant difference between the pH-modified media treatments for both control wells and BAPTA treated wells. This correlates with the data shown in Figure 6.33. This suggests that external Ca^{2+} is not used to a great extent for the secondary response to addition of pH-modified media.

Incubation with TMB-8 prior to addition of pH-modified media showed a dose-dependent decrease in the secondary response, suggesting that most Ca^{2+} comes from

intracellular stores for this response (Figure 6.36). As can be seen, concentrations ≥ 25 μM TMB-8 almost completely inhibit the secondary response. Other work using TMB-8 was carried out using a concentration of 25 μM. Figure 6.36 shows the response to addition of media of pH7.6 with varying concentrations of TMB-8. Addition of control media is also shown, with and without TMB-8 at an effective concentration. As described previously, TMB-8 showed no effect upon the touch response observed upon addition of iso-osmotic, iso-basic media (Figure 6.23). There was also no difference in the initial $[Ca^{2+}]_c$ response with pH-modified media. The inhibition of the secondary response observed in the presence of TMB-8 was seen in all the pH-modified media shown in Figure 6.32 (data not shown).

Figure 6.36: Effect of TMB-8 upon $[Ca^{2+}]_c$ response to pH-modified media



Key: wells pre-incubated for 5 min with 25 μl VS (plus appropriate concentration of TMB-8) prior to measurement. Media added at $t = 1$ min. n = 12, error bars are ±SD.

Statistically, there was no significant difference between inhibition of the $[Ca^{2+}]_c$ response by 25 μM and 50 μM TMB-8, and no difference between the effects of 0 μM and 10 μM TMB-8 treatment upon addition of pH 7.6 media (determined using

a single factor ANOVA and determining the 5% LSD for the period t = 2-3 min; data not shown).

6.5.2 Conclusion

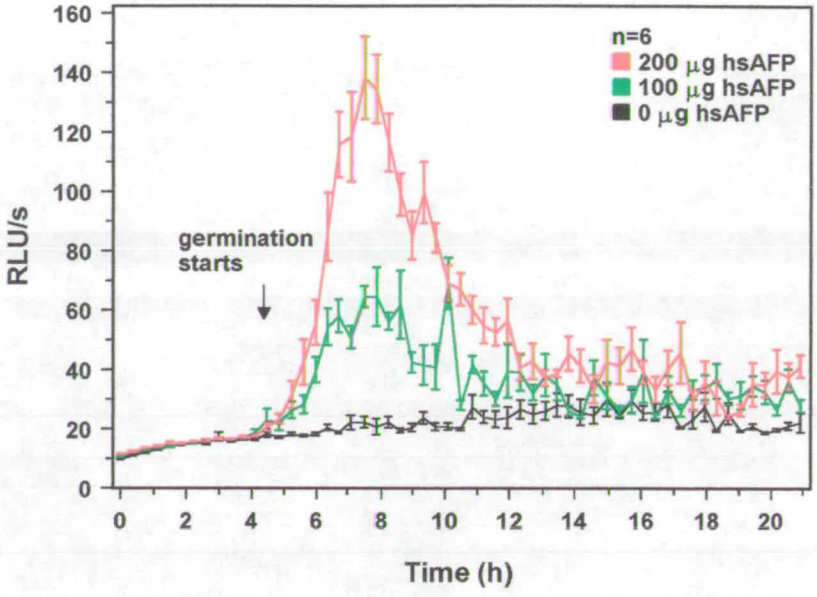
Addition of pH-modified media has been shown to result in an independent secondary response approx. 1 min after the initial mechanical response. In contrast to the normal media responses, the secondary pH response has been shown to require Ca^{2+} from internal stores through TMB-8 sensitive Ca^{2+} channels. Further investigation using more specific intracellular Ca^{2+} channel antagonists (*e.g.* ryanodine) may allow more precise identification of particular stores for this $[Ca^{2+}]_c$ response.

6.6 Effects of antifungal compounds

Zeneca Agrochemicals supplied two antifungal proteins for analysis of their effects upon $[Ca^{2+}]_c$ in *Aspergillus*. The working concentrations of both proteins were given. The first protein, hsAFP was isolated from seeds of *Heuchera sanguinea* (Osborn *et al.*, 1995) is known to affect $[Ca^{2+}]_c$. The second, ACEAMP1 was not thought to involve modulation of $[Ca^{2+}]_c$ (S. Palmer, pers. comm.).

Conidia were germinated in the presence of hsAFP and the luminescence monitored during germination and growth (Figure 6.37). As can be seen, at the onset of germination (approx. t = 6 h, see Figure 6.5) there is a sharp rise in $[Ca^{2+}]_c$ in a dose dependent manner in the presence of the antifungal proteins. In the presence of hsAFP, this falls back to the control basal level luminescence after approx. 6 h.

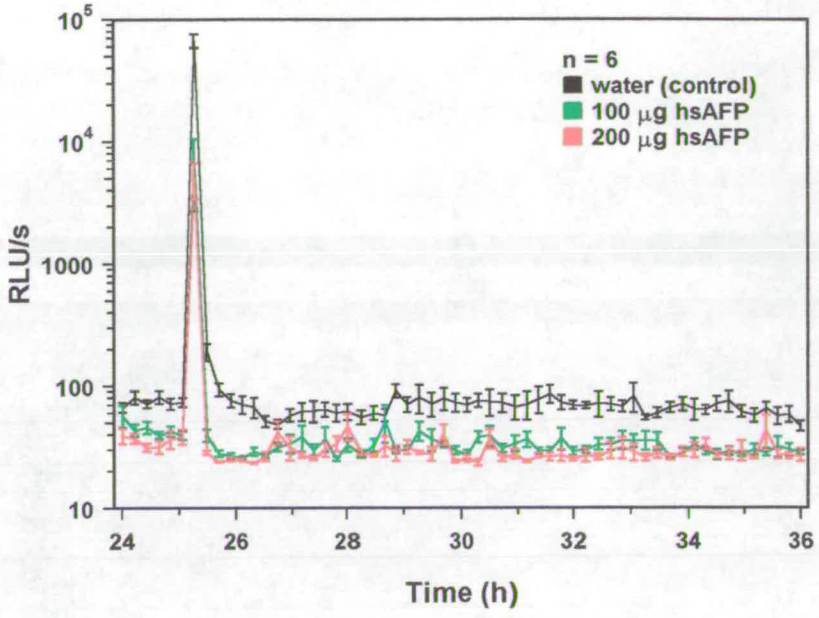
Figure 6.37: Incubation and germination in the presence of hsAFP



Key: hsAFP resuspended in H_2O, and a final vol. of 25 µl added to each well (25 µl H_2O added to control wells) to give the final quantities shown. Cultures incubated from inoculation at 30 °C whilst monitoring luminescence. n = 6, error bars are ±SD.

Continuation of incubation in the presence of hsAFP showed a slight inhibition of growth, as can be seen by the lower basal luminescence levels observed in 24 h cultures (Figure 6.38). Stimulation of the cultures by the addition of 100 mM $CaCl_2$ showed a normal response which was slightly smaller than that obtained for the control, which is possibly due to the amount of mycelia present (Figure 6.38). It is also possible that the results reflect a greater consumption of aequorin in the hsAFP-incubated cultures. The difference between the control and the hsAFP-incubated wells is significant (Figure 6.39). This data verifies what was already known about hsAFP, but does not clarify any further upon the mode of action of the protein (S. Palmer, pers. comm.).

Figure 6.38: Addition of $CaCl_2$ to hsAFP-incubated cultures



Key: Continuation of measurement from Figure 6.37. Wells injected with 100 µl 100 mM $CaCl_2$ at 25 h 12 min. n = 6, error bars are ±SD.

Figure 6.39: Tabular representation of significance of Ca^{2+} response shown in Figure 6.38

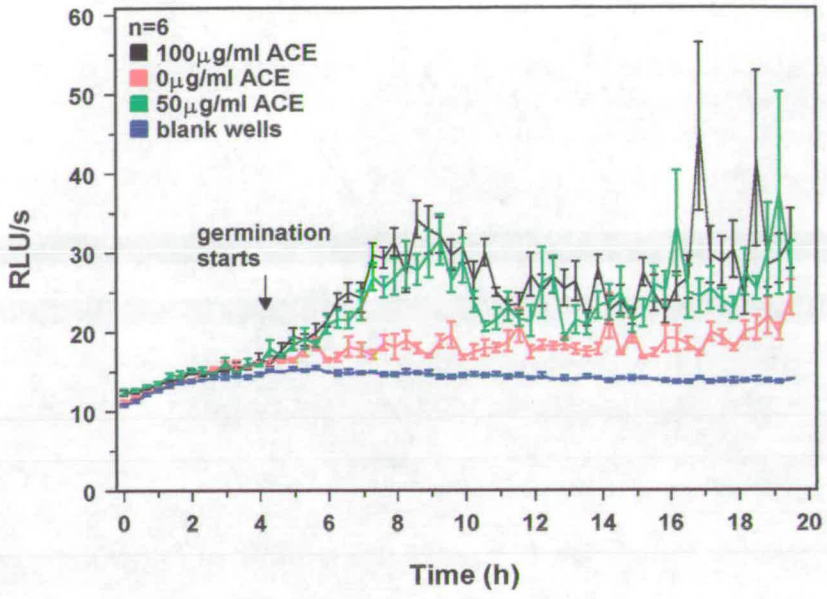
control	100ug hsAFP	200ug hsAFP
66716	6561.7	5036.5

Key: Mean RLU/s shown for peak obtained upon addition of Ca^{2+} in Figure 6.38. 5% LSD = 16,837.4

Incubation of cultures from the time of inoculation with ACEAMP1 caused the $[Ca^{2+}]_c$ level to rise at the onset of germination (Figure 6.40) and stay significantly higher than the control basal level (Figure 6.41).

ACEAMP1 did not have any effect upon response to stimuli such as addition of media or Ca^{2+} (data not shown). Also, the increased basal $[Ca^{2+}]_c$ did not appear to affect growth of the mycelia. Microscopic analysis was not carried out, but cultures conidiated at the correct time and at the usual density.

Figure 6.40: Incubation and germination in the presence of ACEAMP1



Key: ACEAMP1 resuspended in H_2O, and a final vol. of 25 µl added to each well (25 µl H_2O added to control wells) to give the final concentrations shown. Cultures incubated from inoculation at 30 °C whilst monitoring luminescence. n = 6, error bars are ±SD.

Figure 6.41: Tabular representation of significance of data from Figure 6.40

	100 µg ACE	50 µg ACE	0 µg ACE	blank	5% LSD
8-10 h	218.3	200.3	127.5	102.5	9.1
14-16 h	171.5	167.2	130.7	98.3	10.9

Key: Mean RLU/s shown for the periods 8-10 h and 14-16 h for each treatment (Figure 6.40). 5% LSDs shown in final column. Underlined means represent non significantly different datasets.

Further work was not carried out with either of the antifungal proteins.

6.7 Discussion

Using recombinant apoaequorin, a method has been developed to allow successful and routine measurement of $[Ca^{2+}]_c$ in filamentous fungi. The amount of aequorin produced in this study allowed the measurement of basal Ca^{2+} levels during growth

of cultures, and could measure very small changes in $[Ca^{2+}]_c$. The assumption is made that the luminescence changes witnessed are in fact reporting $[Ca^{2+}]_c$. The data presented in this chapter suggest that the luminescence measurements obtained are measuring calcium levels (such as the increased luminescence in the presence of excess $CaCl_2$ and the decreased luminescence reported in the presence of Ca^{2+} chelators). Experiments were not carried out to determine whether aequorin was present in organelles. This could be ascertained by testing for aequorin in the insoluble fraction obtained in whole cell soluble protein preparation.

Using the system developed in this chapter, mycelial responses to several stimuli have been investigated. From the characterisation of the *Aspergillus* transformants, active aequorin is likely to be present in the cytosol. It is also probably in the nucleus, as aequorin is small enough to pass through nuclear pores. This could be avoided by producing a fusion gene for expression only in the nucleus or cytosol. Fusion to a highly expressed native cytosolic protein or to GFP would produce a protein that was too large to pass through the nuclear pores and therefore would accurately measure only $[Ca^{2+}]_c$.

Data obtained using Ca^{2+} channel inhibitors has allowed the responses to several external stimuli to be characterised to a certain extent. This is summarised in Table 6.5. The data obtained showed that immediate responses (within the first few seconds) to any of the stimuli investigated required an external Ca^{2+} source. A secondary increase in $[Ca^{2+}]_c$ was obtained upon addition of pH-modified media. This occurred approx. 1 min after addition, and was shown to not require external Ca^{2+}, but instead be dependent upon internal Ca^{2+} stores.

Table 6.5: Summary of the effects of various inhibitors and modulators on $[Ca^{2+}]_c$ response to the stimuli investigated in *Aspergillus*

	high $[Ca^{2+}]_{EXT}$ response	mechanical stimulus	hypo-osmotic stimulus	external pH change	unstimulated basal $[Ca^{2+}]_c$ level
$[Ca^{2+}]_{EXT}$	n/a	agonist	n.d.	n.d.	nsd (3 mM)
La^{3+}	I (10-20 mM)	n.d.	n.d.	n.d.	n.d.
nifedipine	I (10-25 μM)	no I (25 μM)	no I (25 μM) [1]	no I (25 μM)	n.d.
ryanodine	I (160 nM)	n.d.	n.d.	n.d.	n.d.
TMB-8	no I (25 μM)	no I (25 μM)	no I (25 μM)	I (25 μM)	increases $[Ca^{2+}]_c$ [2]
CPA	I (10 μM)	I (1-10 μM)	I (1-10 μM)	n.d.	increases $[Ca^{2+}]_c$ [2]
EGTA/ BAPTA	n.d.	I (0.5 mM BAPTA)	I (0.5 mM BAPTA)	nsd (0.5 mM BAPTA)	decreases $[Ca^{2+}]_c$ (50 mM EGTA)
Br-A23187	Ag (10-20 μM)	Ag (10-20 μM)	n.d.	n.d.	nsd (20 μM)
caffeine	n.d.	Ag (1mM)	Ag (10 mM)	n.d.	n.d.

Key Stimuli responses investigated shown in columns with inhibitors/ modulators used and their effects shown in rows. n/a; not applicable: n.d.; not determined: nsd; no significant difference (at concentrations stated): I; inhibition (at concentrations stated): no I; no inhibition (at concentrations stated): Ag; agonist (at concentrations stated). [1] possibly slower rate of response. [2] data not shown for long term increase in $[Ca^{2+}]_c$.

Evidence for the involvement of intracellular Ca^{2+} stores in the immediate responses is mixed. Inhibition of the $[Ca^{2+}]_c$ response to addition of external Ca^{2+} was obtained with ryanodine but not with TMB-8. Depending upon the specificity of TMB-8, this may mean that intracellular Ca^{2+} stores are involved in this response. In plant cells, TMB-8 has been shown to be specific to IP_3-sensitive vacuolar Ca^{2+} channels (Johannes *et al.*, 1992b; Schumaker and Sze, 1987) which are insensitive to ryanodine (Muir *et al.*, 1997). In mammalian cells, IP_3-sensitive Ca^{2+} channels and ryanodine receptors are both found in the ER, and are sensitive to TMB-8 and ryanodine respectively (Miller, 1988; Kiang, 1994; Liu *et al.*, 1997). Little is known about the effects of TMB-8 and ryanodine in filamentous fungi, although 50 μM nifedipine has been reported as having no effect upon Ca^{2+} uptake in *N. crassa* (Corzo and Sanders, 1992). This is a higher concentration of ryanodine than that

which showed inhibition (10 μM) in this study. Also, TMB-8 has been shown to inhibit known Ca^{2+}-dependent signalling pathways in *Zoophthora radicans* (Magalhães *et al.*, 1991) and has been shown to only affect intracellular Ca^{2+} stores in *P. parastica* (Warburton and Deacon, 1998). This study also demonstrates that TMB-8 only affects intracellular Ca^{2+} stores, as the mechanical response is not affected by TMB-8 and this was shown to require external Ca^{2+}, whereas the secondary response to pH change required intracellular Ca^{2+} but not external Ca^{2+}. To have confidence in the inhibition obtained with ryanodine in response to external Ca^{2+} at least one other inhibitor must be used to show the same effect. Ruthenium red is a possible candidate. Also, demonstration of any possible differences in TMB-8 and ryanodine-sensitive Ca^{2+} pools in *Aspergillus* is desirable to validate the data. Further analysis of the secondary response to pH change could show this if there are differences in inhibition with TMB-8, ryanodine and ruthenium red. Consumption of active aequorin throughout the individual experments was not taken into account in this study. Repeated stimulation of cultures with $CaCl_2$ at the end of some experiments demonstrated that there was a large excess of active aequorin. However this data was not shown. This will have to be taken into account to successfully determine values for $[Ca^{2+}]_c$.

The concentrations of inhibitors used to provide inhibition in this study are comparable with the concentrations used by other workers with different organisms. The exceptions to this are ryanodine and La^{3+}, both of which were used at higher concentrations than those published. Ryanodine was used at 160 nM in this study, which is twice that used by other workers in filamentous fungi (Corzo and Sanders, 1992), and four times that used to produce 50% inhibition in plant cells (Muir and Sanders, 1996). The marked inhibition observed with ryanodine in this study suggests that the IC_{50} is at a lower concentration than that used, and therefore may be more in line with the published concentrations. Published La^{3+} concentrations are usually around 1 mM, which has been shown to cause 70% inhibition in *Zea mays* plasma membrane Ca^{2+} channels (Marshall *et al.*, 1994) and 93% inhibition in *N.*

crassa Ca^{2+} uptake (Corzo and Sanders, 1992). However, the concentration of La^{3+} required to cause inhibition of the gravitropic response in *A. majus* was between 10-30 mM (Friedman *et al.*, 1998), which correlates with this study. Further work in filamentous fungi is required to characterise the efficacy and specificity of the inhibitors employed in this study.

The $[Ca^{2+}]_c$ changes investigated in this study are relevant to possible physiological changes, with the exception of the external Ca^{2+} response. 50 mM $CaCl_2$ is far too high to be regarded as a typical environment for mycelial cultures. This stimulus was investigated because it was used to isolate high expression transformants, due to the large response and reproducibility of the result. The downstream effects of the $[Ca^{2+}]_c$ changes have not been determined for any of the stimuli investigated. Detailed analyses with other inhibitors (*e.g.* CaM inhibitors), $[Ca^{2+}]_c$ modulators (*e.g.* cADP ribose and IP_3) microscopic analysis for morphological changes (*e.g.* hyperbranching) and gene expression studies could help shed light on the cellular responses.

The luminometry results presented in this thesis are all given as Relative Light Units, which is the output from the luminometer. None of the data has been converted into actual $[Ca^{2+}]$. This is due to the fact that to determine actual $[Ca^{2+}]$ requires ending the experiment with the discharge of all remaining aequorin so that the total amount of aequorin present during the experiment can be determined. This has proved remarkably difficult in filamentous fungi. It has been achieved in *N. crassa* germinating conidia, using addition of 20% acetone and 200 mM $CaCl_2$ (Collis, 1996). The acetone present in this treatment does quenches aequorin luminescence, which has to be taken into account when determining total aequorin discharge. This treatment could not be utilised with the luminometer used in this study, due to the injectors being automatic and not being able to control such a concentration of solvent. Also, growing mycelia are constantly producing apoaequorin, and the only way to limit active aequorin production without disrupting the cultures would be to

remove coelenterazine. This was not done with any of the work shown in this thesis. The alternative method would be to use the coelenterazine analogue, *e* coelenterazine, which produces a bimodal luminescence and therefore can be used to directly quantify $[Ca^{2+}]$. However, with the luminometer used in this study, it was not possible to measure two sets of wavelengths at the same time. Consequently, values for $[Ca^{2+}]_c$ were not determined in this study.

7. FUTURE WORK

The possible areas of research that could be undertaken with the recombinant apoaequorin method developed in *Aspergillus* are many. To continue the research given in this thesis, the following areas would be of interest:

- A more in-depth study with the inhibitors used in this thesis on the $[Ca^{2+}]_c$ responses to the stimuli investigated. Also, development of a method of $[Ca^{2+}]_c$ calibration and determination of the localisation of active aequorin.

- Use of other Ca^{2+} modulators (*e.g.* CaM antagonists, cADP ribose and IP_3) to further analyse the signal transduction components which resulted in increased $[Ca^{2+}]_c$ in response to the stimuli investigated in this thesis.

- Use of mutants compromised in Ca^{2+} signalling and Ca^{2+} homeostasis (*e.g. A. niger* mutants 12/1 and 7/9, two Ca^{2+}-ATPase mutants constructed at TNO, The Netherlands) to further dissect components of the Ca^{2+} signalling pathways analysed in this thesis.

- Analyse $[Ca^{2+}]_c$ responses in mycelia to several other stimuli, such as light, temperature, sugar and nitrogen starvation and response to fungal mating type pheromones.

Further investigations into $[Ca^{2+}]_c$ regulation could be obtained by producing several expression vectors for *Aspergillus*:

- Produce an apoaequorin fusion vector to allow production of a cytosol-targetted aequorin protein that cannot enter the nucleus.

- Analyse variation in aequorin production during growth under *gpdA* promoter by producing apoaequorin expression cassettes with different promoters.

- Analyse the role of organellar free Ca^{2+} by producing targetted apoaequorin expression vectors.

- Production of a GFP::apoaequorin fusion to localise aequorin within the mycelia by fluorescence microscopy.

It is also desirable to be able to image growing mycelia at a magnification high enough to distinguish individual hyphae and measure $[Ca^{2+}]_c$ at the subcellular level. Finally, conversion of luminescence values obtained into actual $[Ca^{2+}]$ is very desirable.

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