Tagging Pathogenicity Genes in the Interaction of Barley and the Fungal Pathogen, *Rhynchosporium secalis*.

by

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Abstract

The purpose of this study was to identify pathogenicity genes in the fungal pathogen of cultivated barley, *Rhynchosporium secalis*. Pathogenicity genes are described as genes that are critical for the successful invasion and colonisation of the host plant but not necessary for life cycle completion in culture. To identify genes a pool of insertion mutants was generated.

Insertional mutants were generated by two methods, restriction enzyme-mediated integration (REMI) and *Agrobacterium tumefaciens*-mediated transformation (ATMT). A detailed REMI study showed circular pAN7-1 vector produced higher transformation efficiencies than linear vector at all enzyme levels tested. Fungal strain 5, in combination with 20 units of the restriction enzyme *Bam*HI produced the highest observed transformation efficiency with approximately 40% of these mutants producing simple, single integrations based on interpreted Southern data. The addition of *Bam*HI increased transformation efficiency at all enzyme levels tested with the exception of the highest enzyme concentration: 200 units of enzyme/transformation reaction. In comparison to REMI, the ATMT protocol proved more efficient than REMI and the binary vector backbone pPZP200 produced >50% simple single copy integrations, interpreted from Southern data. This study is the first ATMT protocol for *R. secalis* and was successfully adapted from other fungal species.

In total, 534 *Bam*HI and *Hind*III REMI mutants of *R. secalis* fungal strain UK7 (83) and strain 5 (453) were screened on the universally susceptible barley cultivar Sloop yielding 10 non-pathogenic mutants, eight from strain 5 and two from UK7, respectively.

During screening experiments strain 5 mutants failed to produce enough spores for a spore suspension to be prepared and inoculated. Strain 5 loses the ability to sporulate after four generations, or successive subculture steps. The inability to sporulate was not correlated to an observable, macroscopic loss in fungal biomass. Starvation experiments utilising carbon and nitrogen sources did not alter sporulation in the sporulating strain 5 sample or reverse the loss of sporulation. However, an overall trend was observed in the sporulation of strain UK7 where sporulation decreased with increasing nitrogen and increased with increasing carbon.

Genomic sequence flanking the integration site was isolated and analysed from six of the ten non-pathogenic mutants. Four putative genes were identified with integrations located in their putative promoter sequences. Sequence similarity searches showed three of these putative genes had similarities to amino acid permeases, cytochrome p450 and rhomboid-like genes. The two putative genes with similarities to amino acid permease and cytochrome p450 genes were selected for targeted gene disruption studies using homologous recombination (HR).

ATMT was used as the delivery system for the HR construct in an attempt to generate a disruption mutant and prove gene function. Over 200 mutants transformed with the two knock out vectors were screened. However, gene disruption experiments failed and could not be repeated due to a lack of resources and time.

In conclusion, this study has demonstrated that the REMI transformation technique is feasible for gene disruption studies in *R. secalis*. Furthermore, ATMT is a viable alternative transformation method that, for future studies, would be the preferable technique.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

Shae Yuill

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Abbreviations

-wk	Week
-d	Day
h	Hour
min	Minute
sec	Second
ml	Millilitres
µl	Microlitres
g	Grams
mg	Milligrams
µg	Micrograms
(w/v)	Weight/volume
M	Molar
mM	Millimolar
mm	Millimetres
nm	Nanometres
UV	Ultraviolet
V	Volts
rpm	Revolutions per minute
REMI	Restriction enzyme-mediated integration
ATMT	*Agrobacterium tumefaciens*-mediated transformation
bp	Base pairs
n.d.	No data
NHEJ	Non-homologous end joining
PPS	Protruding single strand
ORF	Open Reading Frame
HR	Homologous recombination
RT-PCR	Reverse transcriptase-polymerase chain reaction
TAIL-PCR	Thermal asymmetric interlaced-polymerase chain reaction

PSS	Protruding single strand
BLAST	Basic logical alignment search tool
PDA	Pisatin demethylase
EST	Expressed sequence tag
GFP	Green florescent protein
gpdA	*gpdA* promoter
Amp	Ampicillin antibiotic resistance gene
hph	Hygromycin antibiotic resistance gene
Spec	Spectinomycin antibiotic resistance gene
Kan	Kanamycin antibiotic resistance gene
LB	Left border
RB	Right border
MAMPs	Molecular-associated molecular patterns
PAMPs	Pathogen-associated molecular patterns

Chapter 1: Introduction

Leaf scald (or blotch), caused by the fungus *Rhynchosporium secalis*, is a common disease of cultivated barley (*Hordeum vulgare* L.) in many of the crop's major growing regions (Shipton *et al.,* 1974, Tekauz, 1991). Scald is especially prevalent in cooler, semi-humid barley growing areas and, consequently, is an economically important pathogen in Australia (Brown, 1985) where yield reductions can reach up to 40% (Shipton *et al.*, 1974). Given scald's economic importance, there is a great deal of commercial interest in the methods that are used to control the disease.

The following literature review discusses the *R. secalis* pathogen, pathogen variability, symptoms of infection, the infection process, the susceptible and resistant plant host response post-infection and methods of controlling the disease. Following these sections pathogenicity genes are defined and discussed, the methods of identifying pathogenicity genes in fungal plant pathogens are explored and how these methods could be applied in *R. secalis* examined.

1.1 The Pathogen

Scald was first described by Oudemans (1897, cited in Caldwell, 1937) on rye (*Secale cereale*) in The Netherlands and named *Marsonia secalis* Oud. Frank (1897, cited in Caldwell, 1937) isolated the fungus from both rye and barley in Germany. Following Heinsen (1900, cited by Brooks, 1928) transferring the fungus to the genus *Rhynchosporium* because of it's beaked spores, Davis (1922) re-named the fungus as *Rhynchosporium secalis* (Oud.) Davis, according to the International Rules of Nomenclature. No sexual cycle of *R. secalis* has been observed and, therefore, the fungus has been classified as a Deuteromycete (Goodwin *et al.*, 1994).

1.1.1 Pathogenic Variability

Despite the lack of a known sexual cycle, populations of *R. secalis* are generally genetically highly variable (Brown, 1985; McDermott *et al.*, 1989; Burdon *et al.*, 1994; Jorgensen and Smedegaard-

Petersen, 1995). Several different approaches were used to analyse the variability among fungal populations. These approaches include pathogenicity tests (Goodwin *et al.*, 1994; Jorgensen and Smedegaard-Petersen, 1995), isozyme genotyping (Goodwin, 1994), analysis of colony colour, ribosomal DNA (McDermott *et al.*, 1989) and genomic restriction fragment length polymorphism markers (McDonald *et al.*, 1999; Salamati *et al.*, 2000).

Variation among field populations of *R. secalis* occurs within relatively small areas. McDonald *et al.* (1999) reported that the majority of gene diversity within Australian populations of scald occurred within sampling site areas of ~1m^2. Furthermore, within short periods of time, the virulence structure of *R. secalis* populations may change significantly (Zhang *et al.*, 1992).

1.1.2 Disease Symptoms

The visual symptoms of scald vary depending on the severity of the infection. Scald lesions initially present as dark blue-grey areas on the leaf that eventually become necrotic. These necrotic areas develop brown-black borders giving the characteristic scald spot. Disease symptoms appear primarily on the sheaths and blades of leaves. However, in severe cases, glumes, awns and embryos may also be infected (Jenkins and Jemmett, 1967; Habgood and Hayes, 1971). In infected leaves, a significant amount of sporulation occurs in areas where no visual signs of symptoms are apparent (Davis and Fitt, 1990; Lehnackers and Knogge, 1990; Howlett and Cooke, 1992). Hence, *R. secalis* infections could be underestimated where only the visual symptoms of the disease are taken into account.

1.1.3 The Infection Process

All plant diseases are influenced by the environmental conditions present at the time of infection. For *R. secalis* the most influential factors are the number of conidia, the temperature and the humidity. Scald is primarily spread by splash dispersal of conidia (Ayesu-Offei and Clare, 1970; Fitt *et al.*, 1989; Salamati *et al.*, 2000). Favourable conditions for infection are temperatures greater than 10°C and high humidity (>90%) (Polley, 1971). In glasshouse and laboratory studies, temperatures ranging between 15°C and 17°C are generally used to promote infection (Ayesu-Offei and Clare, 1970; Lehnackers and Knogge, 1990; McDonald *et al.*, 1999).

Prior to 1970, reports detailing the infection process of *R. secalis* were contradictory. Mackie (1929) reported that *R. secalis* entered the leaf via stomatal pores, whereas Caldwell (1937) stated that the fungus directly penetrates the leaf cuticle and epidermis to enter the host. Ayesu-Offei and Clare (1970) clarified the method of infection when they reported that infection took place by direct penetration of the cuticle. Most conidia (>80%) germinate within the first 24 hours post-inoculation (Lehnackers and Knogge, 1990). Following germination, two germ tubes are produced from the surface of the conidia, one from each of the two genetically identical cells. Appressoria form at the apices of germ tubes and infection hypha penetrate the cuticle (Ayesu-Offei and Clare, 1970; Jorgensen *et al.*, 1993).

1.2 Post Penetration - The Host Plant Response

One of the most important factors in the capacity of a fungus to successfully develop and reproduce within a host plant is the presence or absence of genes in the plant capable of conditioning resistance to the invading pathogen. Host plant resistance to scald is controlled by single resistance (*R*) genes (Jorgensen, 1992) in an interaction consistent with the gene-for-gene hypothesis (Flor, 1956). This hypothesis states that plant resistance relies on the presence of specific *R* genes in the plant and corresponding specific avirulence (*Avr*) genes in the pathogen (Flor, 1956). The current biochemical interpretation of the 'gene-for-gene' interaction states that the *Avr* genes produce elicitors that directly or indirectly interact with the *R* gene products in the host (Keen, 1990; Staskawicz *et al.*, 1995; Nimchuk *et al.*, 2003; Chisholm *et al.*, 2006). The interaction between the products of *Avr* and *R* genes is assumed to trigger a signal transduction cascade that results in the activation of an array of defence reactions in the host preventing pathogen infection (Hammond-Kosack and Jones, 1996). If either or both of the required components (*R* and/or *Avr* genes) are not present during the interaction, a susceptible reaction results and the pathogen is able to develop (Table 1.1).

Table 1.1: The gene-for-gene hypothesis. In order for a potential host to be resistant against an attacking fungal pathogen the pathogen must contain and express the necessary avirulence gene and the host plant must contain and express the corresponding resistance gene.

		Plant Gene	
		r	*R*
Pathogen Gene	*avr*	S	S
	Avr	S	R

R/*r* – Dominant/recessive resistance gene, respectively

Avr/avr – Dominant/recessive avirulence gene, respectively

S - Susceptible reaction causing infection and disease

R - Resistant reaction that prevents disease

1.2.1 Susceptibility

Following penetration of a susceptible host plant, subcuticular hyphae grow between epidermal cells and branch profusely (Lehnackers and Knogge, 1990). Able (2003) reported the production of significant levels of reactive oxygen in the form of superoxide by epidermal cells associated with fungal invasion. Superoxide production was observed in an early (2 h post inoculation) and a late (21 and 29 h post inoculation) burst. The late superoxide burst of was roughly correlated to subcuticular hyphal growth between epidermal cells. Microscopic studies have shown that epidermal cells in the vicinity of hyphae swell, causing a loss of rigidity of anticlinical epidermal walls eventually leading to epidermal cell collapse (Ayesu-Offei and Clare, 1970; Jones and Ayres, 1972; Lehnackers and Knogge, 1990). Within the first few days post-inoculation, a mean increase in stomatal aperture is observed contributing to an increase in transpiration and the accumulation of root solutes (Ayres, 1972; Ayres and Jones, 1975). Furthermore, an increase in the permeability of epidermal cells is observed in the early stages of infection in susceptible barley leaves (Jones and Ayres, 1974).

A few days after epidermal cell collapse the underlying mesophyll cells collapse. Subcuticular mycelia continue growing to form a subcuticular stroma. As this structure enlarges, the cuticle is separated from the epidermis (Ayesu-Offei and Clare, 1970; Lehnackers and Knogge, 1990). Once the leaf tissue is heavily degraded, during the very late stages of pathogenesis, fungal hyphae can be found growing between the mesophyll cells (Lehnackers and Knogge, 1990).

Sessile conidia develop on short segments of subcuticular hyphae until maturation when conidia tend to become erect and force their way through the cuticle (Caldwell, 1937; Ayesu-Offei and Clare, 1970). Although conidia are produced in lesion areas, they are primarily observed at hyphal extensions that protrude through the cuticle in healthy looking leaf areas (Lehnackers and Knogge, 1990).

Cell death that occurs during fungal infection and development is not caused by fungal hyphae penetrating cells of the susceptible host. Fungal development is predominantly subcuticular and hyphal growth is extra cellular. To obtain sufficient nutrients to support development within the leaf, fungal secretions kill host cells thereby stimulating nutrient release. A small family of Necrosis-Inducing Proteins (NIP1, NIP2 and NIP3) of low relative molecular mass (<10 kDa) were identified in the filtrates of fungal cultures. These proteins caused necrosis upon injection into barley leaves. Furthermore, during pathogenesis of a susceptible cultivar, the occurrence of NIP1 and NIP3 was correlated with the development of visible necrotic lesions indicating these proteins have a role in killing host cells (Wevelsiep *et al.*, 1991). Wevelsiep *et al.* (1993) described a possible mode of action for NIP1 and NIP3. Both proteins stimulated the activity of the plasmalemma-localised Mg^{2+}-dependent, K^+ stimulated H^+-ATPase by about 60%. From this observation, it was hypothesised that cell collapse is due to the impairment of physiological processes controlled by enzymes sensitive to changes in intracellular ion concentrations or pH changes. Ion concentrations within the cell are controlled by the ATPase-generated electrochemical proton gradient.

1.2.2 Resistance

Barley plants resistant to infection by *R. secalis* show no macroscopically visible symptoms. Penetration and growth of hyphae at the very early stages of infection is similar in both resistant and susceptible barley cultivars leading to the collapse of a few epidermal cells. However, in

resistant plants subcuticular mycelia are not formed and degradation of subcuticular hyphae is observed (Lehnackers and Knogge, 1990).

Resistance of barley to the scald pathogen complies with the gene-for-gene hypothesis. Supportive evidence for this was provided by Hahn *et al.* (1993) who reported US238.1, a fungal isolate of *R. secalis* that secretes NIP1 into culture filtrates, elicited the biosynthesis of pathogenesis related (PR) proteins such as peroxidase transcripts (PR9) and thaumatin-like proteins (PR5) in the resistant barley cultivars Turk and Atlas 46, which contain the *R* gene *Rrs1*. PR proteins are components of the plant defence response (for review see Hammond-Kosack and Jones, 1996). In contrast, the cultivars Atlas and Hannchen, lacking the *R* gene, either did not accumulate any components of the defence response or accumulated them later and at a significantly lower level (Hahn *et al.,* 1993). Rohe *et al.* (1995) performed further studies on the *nip*1-*Rrs1* gene interaction. NIP1 protein, when co-inoculated with spores from a fungal race virulent on plants containing the *Rrs1* gene, converted the phenotype of the interaction from compatible (host susceptibility) to incompatible (host resistance). In addition, Rohe *et al.* (1995) transformed the race AU2, normally virulent on *Rrs1* cultivars, with the *nip1* gene yielding avirulent transformants. Furthermore, a fungal mutant in which the endogenous *nip1* gene was replaced by a non-functional gene through homologous recombination was virulent on the *Rrs1* cultivar. These results proved that NIP1 is necessary and sufficient to elicit *Rrs1*-mediated resistance. The gene replacement mutant, in addition to being virulent on *Rrs1* plants, showed a lower degree of virulence on susceptible plants as compared to the wild-type suggesting that the gene product also has a role in fungal virulence (Knogge, 1996). This was one of the first plant-pathogen interactions where a resistance gene was found to recognise a pathogen by a virulence factor.

Although the resistance response has been well researched in plants interacting with biotrophic pathogens (Heath, 1998), currently the resistance mechanisms produced in response to necrotrophic pathogen attack are poorly understood. In biotrophic systems, a plant that identifies an attacking pathogen elicits a series of complex physiological and molecular processes at the site of infection. The culmination of these processes is the programmed death of cells specifically located at the site of infection, referred to as the hypersensitive response (Nimchuk *et al*., 2001). One important component of programmed cell death is the production of reactive oxygen species, including but not limited to superoxide. However, in the *R. secalis* resistance response superoxide production declines following one initial burst two hours post inoculation. One possible explanation

for the reduction of superoxide is that necrotrophic pathogens aim to kill plant cells, and hence, the hypersensitive response, as observed in biotrophic pathogens, is an unsuitable defence mechanism (Able, 2003).

1.3 Disease Control

Modern farming practices contribute to the incidence and severity of leaf scald. Studies made in Canada cited intensified cropping, lack of crop rotation, uniform scattering of straw by the thresher and increased use of shallow cultivation as factors that increase the persistence of scald (Skoropad, 1960). Jefferies *et al.* (2000) reported that intensified cropping, stubble retention, increased use of nitrogenous fertiliser, earlier seeding and higher seeding rates contributed to the incidence and severity of leaf scald in southern Australia. Changes in cultivation practices could, therefore, reduce leaf scald in areas where these approaches are used in barley farming. Currently, however, the two main approaches to control leaf scald are the deployment of naturally occurring forms of host plant resistance and the use of fungicides. *R. secalis* is genetically highly variable (section 1.2.1) providing the potential for its rapid adaptation to a changing environment (McDonald *et al*., 1999). Thus the efficacy of fungicides and resistant cultivars to control *R. secalis* is constantly under threat by the pathogen's ability to evolve and overcome these control measures.

1.3.1 Resistant Cultivars

To develop scald-resistant barley cultivars, breeders require adequate sources of genetic resistance. The gene pools of cultivated barley and wild barley possess good sources of resistance to *R. secalis* (Goodwin *et al*., 1990, Abbott *et al*., 1992). However, the natural variability that exists within populations of *R. secalis* has often resulted in single major gene resistance being rendered ineffective within a few years (Jefferies *et al*., 2000). This problem may be overcome by using strategies that breed and select for race non-specific, durable resistance (Cselenyi *et al*., 1998) or by adopting major gene deployment approaches including the use of multilines, cultivar mixes (Jensen, 1952) or gene pyramiding (Nelson, 1978). Abbott *et al.* (2000) suggested, however, that

cultivar mixtures are not reliably effective in controlling scald disease, especially when one component of the mixture is susceptible because this component can provide the pathogen a with disease reservoir increasing the likelihood that a strain virulent on the resistant cultivars develops. Similarly, multilines, near-isogenic lines differing in only their *R* genes, are practically difficult to produce because of long breeding times (Crute and Pink, 1996) and would also provide a disease reservoir if one of the near-isogenic lines was susceptible. Gene pyramiding is a breeding strategy that combines a number of single major resistance genes into a single cultivar (Kloppers and Pretorius, 1997). This breeding strategy is based on the theory that if mutations responsible for virulence to different *R* genes occur independently at different loci within the fungal genome, then the probability is low that several mutations could occur simultaneously at different loci causing multiple virulence (Schafer *et al.*, 1963). Gene pyramiding has been shown to be an effective strategy for the control of a number of diseases (Burdon, 1993) including scald (Abbott *et al.*, 2000). However, problems may be encountered when combining different *R* genes into a common genetic background. Without markers or pathotypes capable of unequivocally recognising different *R* genes, differentiation between *R* genes in a crossing program is difficult (Brown *et al.*, 1996). Furthermore, epistatic effects between *R* genes can result in changes to gene activity (Kelly *et al.*, 1995) compounding problems associated with pyramiding *R* genes.

Although *R* genes provide the most efficient and environmentally friendly means of controlling diseases they are often overcome by their respective pathogen and lose their efficacy (Salamati and Tronsmo, 1997). Consequently, many agricultural systems supplement genetic resistance with other control strategies such as the use of fungicides.

1.3.2 Chemical Control – Fungicides

Fungicides have a significant role in controlling scald of barley (Taggart *et al.*, 1999). Demethylation-inhibiting (DMI) fungicides and the benzimidazole fungicide, carbendazim, are the most commonly used fungicides against *R. secalis* (Taggart *et al.*, 1998). DMI fungicides inhibit the sterol 14α-demethylase step in fungal sterol biosynthesis (Kendall *et al.*, 1993) causing changes to the sterol composition in fungal cells. A qualitative change in sterol composition is likely to alter membrane fluidity (Buchenauer, 1987). Carbendazim binds to tubulin, the protein

subunit of microtubules, causing inhibition of mitosis and other processes involving microtubules (Davidse and Flach, 1978).

The extensive use of some DMI fungicides led to a decline in *R. secalis* sensitivity in the UK during the 1980s (Taggart *et al.*, 1999). Compounding this problem is the use of these fungicides for other pathogens of barley, so that even when scald is not the targeted disease, *R. secalis* populations are subject to selection for fungicide-resistant strains (Kendall *et al.*, 1993). A loss in field performance led to the recommendation that carbendazim should be used in mixtures with those DMI fungicides that retained their effectiveness (Taggart *et al.*, 1999). This proved an ineffective 'anti-resistance' strategy when Locke and Phillips (1995) reported carbendazim resistance in England and Wales in 1992 and 1993. Wheeler *et al.* (1995) sequenced the β-tubulin gene and showed that carbendazim resistance is linked to point mutations within the coding sequence. The resulting amino acid change decreased fungicide binding to β-tubulin.

A consequence of fungicide resistance in fungal populations is the need to produce new fungicides. Thus a number of products with novel modes of action were produced (Gullino *et al.*, 2000). An important aspect of fungicide research is the identification of novel target sites for fungicides. The infection process has many discrete steps, spore attachment to the plant surface, germination, germ tube elongation, appressorium formation and penetration into the plant tissue. Because these steps are unique to fungal pathogens, specific fungicides that target these infection processes or the proteins that control them would have fewer side effects to other non-target organisms (Struck *et al.*, 1998; Dufresne *et al.*, 1998). However, this approach is currently limited by our lack of understanding of the fungus' pathogenicity genes.

1.4 Fungal Pathogenicity Genes

Pathogenicity is defined as the disease-producing capacity of a pathogen. If this literal definition were extended to all genes that could affect the pathogenicity of *R. secalis*, it would include all genes essential for the completion of the life cycle of the fungus. Consequently, essential housekeeping genes that are not directly involved in the infection process would be included in this definition. To clarify the definition of a pathogenicity gene, Schafer (1994) proposed the following:

“A fungal pathogenicity gene is not necessary for the completion of the life cycle and is directly and intrinsically involved in pathogenicity under natural conditions.” However, this definition cannot apply to obligate pathogens because genes responsible for successful infection and disease development cannot be separated from those genes that are essential for the completion of the life cycle (Oliver and Osbourn, 1995). Hence, Kahmann and Basse (1999) suggest a more restrictive designation of pathogenicity genes by eliminating those genes that cause growth defects or defects in development during axenic culture. Schafer (1994) also suggested virulence means the degree of disease expression. Thus, virulence genes do not affect the pathogen’s basic ability to successfully infect and reproduce within a host. Instead, they are responsible for the severity of disease expression.

The number of pathogenicity genes that have been described in the literature (greater than 80) makes it inhibitory to discuss each gene individually (see review, Idnurm and Howlett, 2001). The pathogenicity genes identified can be loosely grouped into two major categories: genes involved in signalling and effector genes that have some physiological function. However, it should be noted that a number of novel genes have been identified that have no known function.

Changes in gene expression occur when fungi are exposed to changes in the environment. Genes that control gene expression during these environmental changes are referred to as signalling genes. Examples of signalling genes essential for pathogenicity are the G protein α subunit *magB* (Liu and Dean, 1997), and the MAP kinases *pmk1* and *mps1* (Xu and Hamer, 1996; Xu *et al.*, 1998) from *Magnaporthe grisea*. Given that signalling genes often control more than one downstream effector gene, it is often difficult to determine which aspect of fungal physiology is responsible for the loss of pathogenicity.

A number of effector genes have been identified from various phytopathogenic fungi. Idnurm and Howlett (2001) classified these effector genes into four groups: genes controlling the production of infection structures, genes encoding enzymes for cuticle and cell wall degradation, genes responding to changes in the host environment and fungal toxin genes. The remainder of this section provides brief examples of genes placed within these four categories.

Appressoria are very typical infection structures produced by most plant pathogenic fungi in the early stages of infection. The grass pathogen *M. grisea* becomes non-pathogenic if appressoria formation is disrupted. One of the critical signalling molecules for the formation of appressoria is cyclic AMP (cAMP). Transmission of the cAMP signal is dependant on protein

kinase A (PKA) activity. Disruption of the gene encoding the catalytic subunit of fungal PKA resulted in delayed appressorium morphogenesis and consequently a loss of pathogenicity (Mitchell and Dean, 1995; Xu *et al.*, 1997).

Enzymatic degradation of a plant's protective barriers such as cutin, pectin and the cell wall may be essential for complete pathogenicity of plant pathogenic fungi. Rogers *et al.* (1994) showed that a loss of cutinase production significantly decreased the *Fusarium solani* f.sp. *pisi* (*Nectria haematococca*) pathogen's ability to infect its host plant, pea (*Pisum sativum*). However, the enzymes responsible for the degradation of plant's physical barriers are often encoded by multigene families or other unrelated genes (see review, Lebada *et al.*, 2001). Hence, inactivity of one gene through disruption can be masked by other genes.

Pathogenic fungi encounter a rapidly changing environment as they begin to colonise a host. Environmental changes include, but are not limited to, exposure to toxic compounds present in plant leaves. A vital mechanism for fungal survival in this hostile environment is the removal of these toxic compounds as they are accumulated in fungal cells. An example of such a fungal mechanism was revealed by the identification of the *ABC1* gene in *M. grisea* (Urban *et al.*, 1999). This gene showed significant similarity to ATP-driven efflux pumps, part of the ABC transporter superfamily of membrane proteins.

Plant pathogenic fungi produce host-specific and host-non-specific toxins to disable host cell function or kill host cells. Host-specific toxins are determinants of both host range and pathogenicity. An example of a pathogenicity determinant comes from *Pyrenophora tritici-repentis* where transformation of $ToxA^-$ isolates with the *ToxA* gene resulted in toxin producing, pathogenic mutants of the fungus on sensitive wheat cultivars (Ciuffetti *et al.*, 1997).

1.4.1 Methods of Identifying Pathogenicity Genes

Several different molecular approaches have successfully identified fungal pathogenicity genes (reviewed in Oliver and Osbourn, 1995; Hensel and Holden, 1996; Gold *et al.*, 2001). These strategies have been possible due to advances made in genetics and molecular biology such as the development of DNA transformation protocols and of selectable markers for fungi.

1.4.1.1 Educated Guessing

Based either on the detailed knowledge of the microbe being studied or on knowledge from a related species "educated guessing" may generate candidate pathogenicity genes (Hensel and Holden, 1996). Confirmation of the suspected function then requires targeted disruption of a gene (Dufresne *et al.*, 1998). For this purpose, a mutant is constructed either by gene disruption or gene replacement and its pathogenicity compared to that of the wild type strain. This strategy was exemplified by Xu and Hamer (1996) who used gene replacement to test whether a MAP kinase was a pathogenicity factor from *M. grisea*. Exogenous cAMP had been shown to be important in appressorium formation and hence, disruption of a gene related to cAMP-dependant protein kinases was found to delay appressoria formation (Mitchell and Dean, 1995). Therefore, Xu and Hamer (1996) designed degenerate primers and cloned a putative MAP kinase (*PMK1*) homolog from *M. grisea*. Genomic clones from the *PMK1* locus were then used to construct a replacement vector and the gene subsequently replaced with a gene coding for hygromycin antibiotic resistance. Knockout transformants failed to produce symptoms on rice and barley where symptoms were observed from the wild type strain, supporting the role of *PMK1* in pathogenicity.

Expression-based strategies have also been used to isolate genes involved in pathogenicity. This method is based on the assumption that pathogenicity genes are expressed during the infection process. One example of an expression-based method was provided by Talbot *et al.* (1993) who cloned the *MPG1* gene from *M. grisea*. This study utilised differential screening of a cDNA library made from heavily infected rice leaves. Replica filters of this library were then probed with labelled cDNAs from the pathogen in culture, and from infected and uninfected rice plants. Many fungal clones hybridised preferentially to the probe from infected plants. The biomass in a leaf contributed by the fungus was then used to estimate the mRNA increase corresponding to these clones. One cDNA clone showing strong induction contained a gene encoding a hydrophobin-like protein (*MPG1*). Deletion of this gene from the wild type strain yielded mutants causing significantly reduced symptoms compared to the wild type strain suggesting *MPG1* to be involved in virulence rather than in pathogenicity.

Differential expression studies have led to several other putative pathogenicity genes being cloned, including *ipi* genes from *Phytophthora infestans* (Pieterse *et al.*, 1993) and the *cap*

genes in *Colletotrichum gloeosporioides* (Hwang *et al.*, 1995). Although expression-based strategies can specifically identify genes induced during infection, problems may arise with this approach because some genes identified by this strategy may not be required for pathogenicity. For example, housekeeping genes that respond to the environment encountered in the host may be triggered. Furthermore, there is the possibility that some pathogenicity genes are not induced during disease development (Dufrense *et al.*, 1998).

1.4.1.2 Mutagenesis

Mutagenesis is an extremely powerful tool in the identification of pathogenicity genes. Mutation-based strategies are separated into two groups, directed mutagenesis such as gene replacement and disruption (previously mentioned in this section), and random mutagenesis. The advantage of using random mutagenesis is that no prior knowledge about the target gene or genes, as is the case with pathogenicity genes, is required (Hensel and Holden, 1996). However, random mutagenesis approaches are limited to fungal species that are at a haploid stage during development within the plant (Dufresne *et al.*, 1998). Exposing the organism to mutagenesis-inducing chemicals, UV light or sources of radiation is used to induce random mutations within genomic DNA. One of the greatest problems with using this strategy is that many mutants generated by random mutagenesis often contain multiple mutations. Although this problem can be resolved by genetic analysis using a crossing program, the sexual stages of many plant-pathogenic fungi have not been observed and therefore genetic separation of multiple mutations cannot always be achieved in the laboratory (Oliver and Osbourn, 1995).

Random insertional mutagenesis using DNA-mediated transformation has several benefits when attempting to isolate pathogenicity genes. Insertional mutagenesis facilitates the cloning of genes of interest because each gene is marked with a molecular tag (Shuster and Bindel Connelley, 1999). There are, potentially, several methods for generating mutants by insertional mutagenesis. Biolistic gene transfer has been attempted in *Aspergillus nidulans* with limited success. Mutants obtained by this process were genetically unstable (Barcellos *et al.*, 1998).

Agrobacterium tumefaciens has been widely used in plant research and gene transfer to plants (Dunn-Coleman and Wang, 1998; de Groot *et al.*, 1998). In the wild, *A. tumefaciens* is responsible for inducing tumors, or crown galls, in plants. During tumor induction, *A. tumefaciens*

transfers part of its tumor-inducing (Ti) plasmid, the T-DNA, to plant cells and the T-DNA is randomly integrated into the plant nuclear genome. T-DNA transfer is dependent on the induction of a set of virulence genes (*vir*) that are also present on the Ti plasmid. Induction of the *vir* genes occurs when the compound acetosyringone is secreted from the wound sites of plants. Once integrated into the nuclear genome, *onc* genes, contained in the T-DNA, are expressed leading to plant cell proliferation and the subsequent formation of a tumor (Bundock *et al.*, 1995; de Groot *et al.*, 1998, reviewed in Zupan *et al.*, 2000). Modified *Agrobacterium* vectors are widely used in plant transformation because the vir system will process and mediate the transfer of any DNA that exists between the 25 bp repeats, called the left and right borders, which normally flank the T-DNA of a wild type strain (Zupan *et al.*, 2000). Recently *A. tumefaciens*-mediated transformation (ATMT) has been extended to include non-plant hosts such as baker's yeast, *Saccharomyces cerevisiae* (Bundock *et al.*, 1995), and the fungal species *Fusarium oxysporum* (Mullins *et al.*, 2001), *Aspergillus awamori*, *Aspergillus niger*, *Fusarium venenatum*, *Trichoderma reesei*, *Colletotrichum gloeosporioides*, *Neurospora crassa, Agaricus bisporus* (de Groot *et al.*, 1998) and *Paecilomyces fumosoroseus* (Lima *et al.*, 2006). Although, at present, no pathogenicity factors have been identified using ATMT, *A. tumefaciens* is potentially a valuable tool for random insertional mutagenesis in plant-pathogenic filamentous fungi. Many of the transformation techniques available for filamentous fungi involve the transformation of fungal protoplasts. Protoplasting fungal spores can be time consuming and may be responsible for unwanted mutations caused by nucleases that contaminate enzymes used in fungal protoplasting (Kahmann and Basse, 1999).

Insertional mutagenesis strategies that enhance the transformation process by increasing transformation efficiency are important for producing mutant populations intended for the purpose of isolating pathogenicity genes. Restriction enzyme-mediated integration (REMI) is a variation of traditional transformation protocols where linearized plasmid DNA is transferred into fungal protoplasts in the presence of a restriction enzyme. REMI is based on the hypothesis that the restriction enzyme responsible for cleaving the plasmid creates corresponding sites in the genome into which the plasmid can integrate (Kahmann and Basse, 1999). This method was originally developed in *S. cerevisiae* where *Bam*HI-restricted DNA was transferred in the presence of the *Bam*HI restriction enzyme resulting in an increase in transformation efficiency (Schiestl and Petes, 1991). Kuspa and Loomis (1992) extended this work by using REMI to transform *Dictyostelium discoideum* and reported that a 20-fold increase in transformation efficiency could be achieved if

linearized plasmid was transformed in the presence of a restriction enzyme. However, the increase in transformation efficiency varies depending on the restriction enzyme used, the applied concentration, the conformation of the vector, the species used for transformation and the transformation protocol applied (reviewed in Maier and Schafer, 1999). Transformation efficiency of REMI compared to other methods was increased up to approximately 20-fold in *Cochliobolus heterostrophus* (Lu *et al.*, 1994), 2-5-fold in *Penicillium paxilli* (Itoh and Scott, 1997), 7-fold in *Coprinus cinereus* (Cummings *et al.*, 1999), 10-fold in *M. grisea* (Shi *et al.*, 1995), 14-fold in *Mycosphaerella zeae-maydis* (Yun *et al.*, 1998) and 2.5-fold in *Ustilago maydis* (Bolker *et al.*, 1995). Furthermore, REMI has also been reported to increase the number of single copy integrations in *Ustilago maydis* (Bolker *et al.*, 1995). Single copy integrations are extremely desirable when attempting to tag genes by insertional mutagenesis due to the ease at which the transformants can be analysed to identify the genomic insertion site.

Using REMI, pathogenicity genes from the fungal pathogens of rice (*M. grisea*; Sweigard *et al.*, 1998), tomato (*Alternaria alternata*; Akamatsu *et al.*, 1997) and maize (*Cochliobolus heterostrophus*; Lu *et al.*, 1994; *Ustilago maydis*; Bolker *et al.*, 1995) have been tagged and cloned. Although this suggests that REMI is an efficient tool for identifying pathogenicity genes, some mutants can contain separate mutations generated by this method, unlinked to the transformed DNA (Balhadere, *et al.*, 1999; Epstein *et al.*, 1998; Sweigard *et al.*, 1998). These untagged mutations are either caused by damage to the fungal genome during protoplasting (Kahmann and Basse, 1999) or by restriction enzyme activity during transformation (Sweigard *et al.*, 1998). Consequently, verification of any putative pathogenicity gene tagged using REMI is required by functional complementation of the wild type gene in the non-pathogenic mutant originally generated (Mullins *et al.*, 2001) or by targeted gene disruption in the wild type isolate.

1.4.2 Tagging Pathogenicity Genes in *Rhynchosporium secalis*

Due to the lack of a sexual stage in *R. secalis* (Goodwin *et al.*, 1994) any functional analysis of genes involved in its interaction with the host, barley, requires transformation-based techniques. Rohe *et al.* (1996) successfully transformed fungal protoplasts produced from *R. secalis* with two plasmids containing dominant selectable markers. The first plasmid, pAN7-1, was originally constructed for the transformation of *Aspergillus nidulans* and contains a dominant selectable

marker, the hygromycin B phosphotransferase gene (*hph*) from *E. coli* mediating resistance to the antibiotic hygromycin (Punt *et al.*, 1987). The second plasmid, pAN8-1, contains the phleomycin antibiotic resistance gene derived from *Streptococcus hindustanus* (Mattern *et al.*, 1988). This plasmid was designed for the transformation, and subsequent selection of *A. nidulans* and *A. niger* transformants.

In order to successfully identify pathogenicity genes in *R. secalis*, an efficient transformation protocol is required. REMI has increased transformation efficiency in a number of filamentous fungi. However, given REMI can be responsible for generating mutations that are unlinked to the transformed DNA, a transformation protocol that minimises the damage to the target organism is desirable. Although ATMT has not been tested or achieved in *R. secalis*, this method has been used in other filamentous fungi. Another advantage of ATMT is that it is a faster less labour intensive protocol. One of the aims of this thesis is to develop both protocols and compare their efficiencies. The second aim is to use the transformants produced to identify pathogenicity genes in *R. secalis*.

Chapter 2: Fungal Transformation (Mutagenesis)

2.1 Introduction

The first step in a gene tagging study is the generation of a pool of insertion mutants. As previously stated, insertion mutants provide excellent experimental material because the mutant phenotype should be linked to the molecular tag inserted into the fungal genome. Generation of a mutant pool is dependent on the transformation protocols available for the organism of interest.

Transformation of the fungus *Rhynchosporium secalis* was first reported by Rohe *et al.* (1996) who introduced hygromycin and phleomycin antibiotic resistance genes via the plasmids pAN7-1 (Punt *et al.*, 1987) and pAN8-1 (Mattern *et al.*, 1988), respectively, using a polyethylene glycol/$CaCl_2$ transformation protocol. However, recent work has reported that fungal transformation in the presence of a restriction enzyme, subsequently named restriction enzyme-mediated integration (REMI), resulted in substantially increased transformation efficiencies (reviewed by Kahmann and Basse, 1999). REMI is based on the hypothesis that the restriction enzyme cleaves the plasmid at a single site and creates corresponding sites in the fungal genome into which the plasmid can integrate (Kahmann and Basse, 1999). The implication in this case is that a lack of sites available for integration of the molecular tag is rate-limiting in transformation (Itoh and Scott, 1997). The increase in transformation efficiency is dependent on a number of different factors including the target organism, the form (circular vs. linearised) of the plasmid used as the tag and the restriction enzyme included in the transformation assay (reviewed by Maier and Schafer, 1999). REMI had been successfully established for *R. secalis* (S. Albert, unpublished), however, no detailed analysis of the effect of enzyme concentration on transformation efficiency has been made. Given the success of REMI in tagging pathogenicity genes from other plant pathogenic fungi (Sweigard *et al.*, 1998, Akamatsu *et al.*, 1997, Lu *et al.*, 1994) this transformation protocol was applied in order to generate a pool of insertion mutants for use in the current study.

Recently, REMI has received significant criticism because a substantial proportion of mutant phenotypes appear to be unlinked to the molecular tag (Sweigard *et al.*, 1998; Balhadere *et al.*, 1999; Linnemannstons *et al.*, 1999). Furthermore, REMI mutants were found to contain

genomic rearrangements such as deletions and duplications (Linnemannstons *et al.*, 1999; Lee *et al.*, 2006). Two critical steps in the transformation protocol that can potentially cause damage to the fungal genome have been identified: digestion of the fungal cell wall for the production of protoplasts and the addition of the restriction enzyme. Digestion of the cell wall requires high concentrations of hydrolytic enzymes that have been linked to genomic damage (Kahmann and Basse, 1999). Furthermore, there is concern that while the restriction enzyme creates an integration site for the molecular tag, its activity at locations unlinked to any integration site may lead to point mutations or genomic rearrangements.

R. secalis is considered an imperfect fungus because no sexual stage has been observed. In fungi exhibiting sexual cycles the genetic cause of phenotypic mutations unlinked to a molecular tag can often be identified using crossing experiments. Since this is not an option in *R. secalis* there was interest in developing different transformation methods. *Agrobacterium tumefaciens*-mediated transformation (ATMT) of filamentous fungi was first reported by de Groot *et al.* (1998) and has since been adapted to a wide variety of fungi (Michielse *et al.*, 2005). ATMT is an attractive alternative to REMI because the two critical steps mentioned above, protoplasting and the addition of a restriction enzyme, are not required. Hence, it is considered likely that an ATMT protocol will cause less damage to the fungal genome during the transformation process. Therefore, the aim of this study was to develop an efficient transformation protocol for *R. secalis* and to generate a pool of *R. secalis* insertion mutants for pathogenicity gene identification. To do this two techniques, ATMT and REMI are compared with regard to transformation efficiency, insertion copy number, complexity and stability in addition to complexity of the transformation protocols in order to evaluate their potential for future tagging studies.

2.2 Materials and Methods

2.2.1 Fungal Strains and Culture Conditions

Three different strains of *R. secalis* were employed in the current study: strains 5 and H2.5, both indigenous to Australia, were provided by Ms Lindy Scott of the Field Crop Pathology Unit, South Australian Research and Development Institute (SARDI). Strain 5 was originally collected at Kingsford, South Australia, from the barley variety Skiff whereas strain H2.5 was isolated from the

variety Atlas at the same location. The third strain, UK7, originated from the collection of the Welsh Plant Breeding Station in Aberystwyth, U.K. (Lehnackers and Knogge, 1990), and was supplied by Dr Wolfgang Knogge, The University of Adelaide, Discipline of Plant and Pest Science. Initially, quarantine restrictions prevented the use of UK7, a proven laboratory strain, and so indigenous isolates of *R. secalis* were selected. Both strains, 5 and H2.5, were considered as the most aggressive strains in the available indigenous collection. Hence, symptoms were observed earlier than other indigenous strains making strains 5 and H2.5 ideal for the identification of non-pathogenic mutants.

For spore isolation, *R. secalis* was grown on Lima bean agar (1.5% w/v) at 18°C and 100% humidity in the dark (Lehnackers and Knogge, 1990). Fungal culture plates were initially started with a minimum of 1 x 10^4 spores. The production of Lima bean agar has previously been described by Williams *et al.* (2003). In brief, 25 g of lima beans were autoclaved for 20 min in 450 ml of H_2O. From the resultant homogenate, 250 ml were decanted through muslin cloth and diluted to a final volume of 1 L. The solution was then combined with 15 g of Bacto Agar (Becton, Dickinson and Company, Singapore) and sterilised by autoclave for 20 min. To ensure the absence of fungicides the Lima beans were obtained from the Organic Market and Cafe, Stirling, South Australia. For the isolation of mycelia, the fungus was cultured in liquid Fries medium No.3 supplemented with 21.91 mM sucrose and 0.1% (w/v) yeast extract (Wevelsiep *et al.*, 1991). Where fungal cultures grown on solid media were transferred to new plates at 10-d intervals, fungal mycelia were harvested after 14-d of liquid culture.

2.2.2 Restriction Enzyme-Mediated Integration (REMI)

2.2.2.1 Preparation of Fungal Protoplasts

The protoplasting protocol used in this study was adapted from Rohe *et al.* (1996) with the following modifications: Fungal mycelia were mechanically dispersed using a DIAX 900 homogeniser (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) for 5-10 sec on speed setting 3; cell wall lysing enzymes used were β-D-glucanase and driselase (Interspex Products Inc, San Mateo, CA, USA). Furthermore, the final protoplast titre was adjusted to 1 x 10^8 protoplasts/ml using a Neubauer counting chamber of 0.1 mm (Hausser Scientific, Horsham, PA, USA).

2.2.2.2 Protoplast Transformation

The protoplast transformation protocol was adapted from Rohe *et al.*, (1996) with the following modifications: To 200 µl of protoplast suspension, 10 µg of circular or pre-linearised pAN7-1 plasmid DNA (Punt *et al.*, 1987) were added with the desired amount (units) of restriction enzyme. All restriction enzymes used in REMI transformations were not affected by methylation. Linear plasmid DNA was produced by digestion with the restriction enzyme *Bam*HI (New England Biolabs, Ipswich, MA, USA). Following digestion, the restriction enzyme was inactivated by adding an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and brief mixing. The upper aqueous phase was recovered by centrifugation for 10 min at 6000 rpm (Biofuge - Pico, Heraeus1[1]). The DNA was then precipitated from the supernatant by the addition of 1.5 volumes of 100% ethanol, 0.1 volumes of 3 M sodium acetate (pH 5.2) and a DNA pellet was formed by centrifugation at 13000 rpm for 10 min. The pellet was washed once in 70% ethanol, air-dried at room temperature and resuspended in an appropriate volume of nanopure water[2]. When the pAN7-1 plasmid had been pre-linearised, the respective restriction enzyme was again added for the transformation.

The mixture of protoplasts, plasmid DNA and restriction enzyme was then incubated on ice for 20 min. Following incubation, three 200 µl aliquots and one 1000 µl aliquot of 50% polyethylene glycol 3350, dissolved in protoplast buffer (10 mM Tris/HCl, 0.7 M KCl, 50 mM $CaCl_2$, pH 7.5) were successively added and mixed with the protoplast suspension before addition of the next aliquot. The protoplast suspension was returned to ice for another 20 min before 10 ml of protoplast buffer were added and the sample centrifuged for 5 min at 4000 rpm (Megafuge1.0, Heraeus). Following centrifugation, the supernatant was removed, the pellet was resuspended in 10 ml of protoplast buffer and again centrifuged for 5 min at 4000 rpm (Megafuge1.0, Heraeus). The supernatant was discarded and the protoplast pellet resuspended in 100 µl of 10x concentrated Fries medium No. 3, supplemented with 21.91mM sucrose and 0.1% (w/v) yeast extract (Wevelsiep *et al.*, 1991), and 1 ml of 1 M sorbitol, 50 mM $CaCl_2$, 10 mM Tris, pH 7.5. Subsequently, the protoplast suspension was incubated at 18ºC for 48 h to allow regeneration before aliquots were plated onto Lima bean agar supplemented with 0.6 M sucrose and 100 µg hygromycin B (Becton, Dickinson and

[1] Unless otherwise stated all centrifuge steps were carried out in the Biofuge – Pico centrifuge.

[2] Unless otherwise stated all nanopure water was used for all aqueous solutions.

Company, Singapore). If negative controls were used, aliquots were plated onto Lima bean agar lacking the antibiotic. REMI mutants were not single-spored. This was deemed unnecessary because transformant colonies were excised from selective plates with very low colony numbers.

2.2.3 *Agrobacterium tumefaciens*-Mediated Transformation (ATMT)

2.2.3.1 Construction of the Binary Vectors

Based on the structure of the binary vectors used by de Groot *et al.* (1998) two binary vectors were constructed. The first vector, pBin19-pAN7-1 (Fig 2.1), contains the binary vector pBin19 (Bevan, 1984; Frisch *et al.*, 1995) and is identical to the vector pUR5750 (de Groot *et al.* 1998). The second vector, pPZP200-pAN7-1 (Fig 2.1), was based on binary vector pPZP200 (Hajdukiewicz *et al.*, 1994). The construction of both vectors is described in the following.

pBin19-pAN7-1: The plasmid pAN7-1 was digested with the restriction enzymes *Bgl*II and *Hind*III (New England Biolabs, Ipswich, MA, USA) to release two fragments of 3987 bp and 2972 bp, respectively. The binary vector pBin19 was digested with the restriction enzymes *Bam*HI and *Hind*III (New England Biolabs, Ipswich, MA, USA) to release two fragments of 30 bp and 11747 bp. The fragments were separated on a 1% agarose/TAE gel [1% (w/v) agarose dissolved in TAE: 40 mM Tris-acetate, 1 mM EDTA, pH 8.0][3], stained with ethidium bromide and visualised under UV light. The 3987 bp fragment from pAN7-1 and the 11747 bp fragment from pBin19 were excised from the gel, purified using the QIAquick® Gel Extraction Kit (Qiagen Pty Ltd, Doncaster, VIC, Australia) and ligated using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). Both *Bgl*II and *Bam*HI create the same 4 bp 5' overhang post-digestion permitting the association of sticky ends and ligation that generates a chimeric *Bgl*II/*Bam*HI restriction site.

pPZP200-pAN7-1: Construction of this binary vector is essentially the same for the pBin19-pAN7-1 vector. The treatment of pAN7-1 is identical (*Bgl*II/*Hind*III). pPZP200 was digested with the restriction enzymes *Bam*HI and *Hind*III (New England Biolabs, Ipswich, MA, USA) to release two fragments of 30 bp and 6711 bp. The 6711 bp fragment was separated on a 1% agarose/TAE gel, stained with ethidium bromide, visualised under UV light, excised from the gel and used in the ligation.

[3] Unless otherwise stated all agarose gels were 1% agarose (w/v) dissolved in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0).

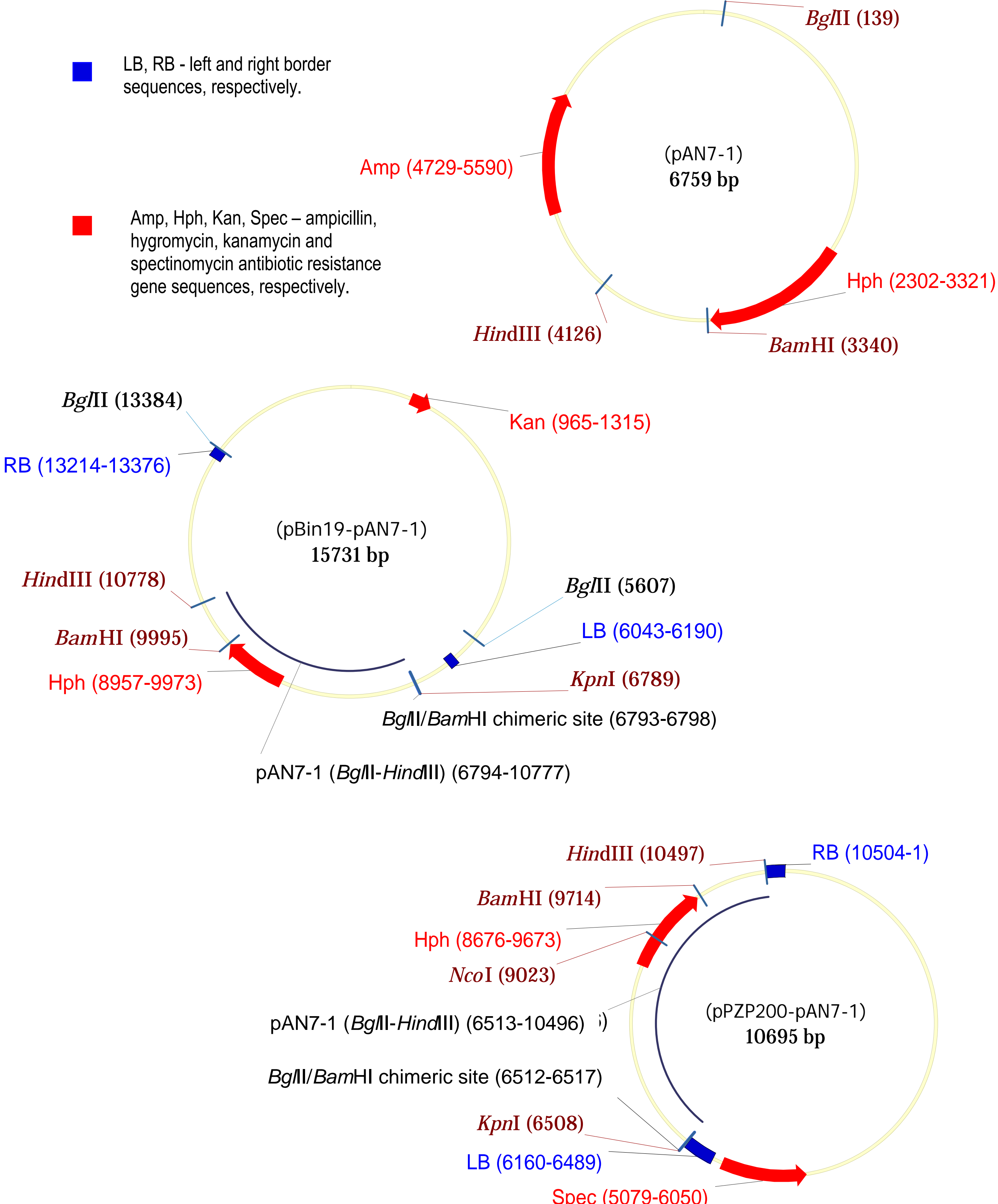


Figure 2.1: Maps of plasmid pAN7-1 and binary vectors pBin19-pAN7-1 and pPZP200-pAN7-1.

2.2.3.2 Amplification of the Binary Vectors in E. coli *Cells*

Competent cells of *E. coli* strain *DH5α* were produced using a method adapted from Chung *et al.* (1989). A single bacterial colony was selected from a Luria-Bertani (LB) agar plate (LB broth: 1.0% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1.0% (w/v) NaCl, pH 7.0: LB Agar: 1.5% (w/v) agar dissolved in LB broth) and incubated in 2 ml of LB broth for 16 h at 37°C with constant shaking at 300 rpm (Orbital Mixer – Incubator, Ratek Instruments, Boronia, Vic., Australia). Subsequently, 500 µl of the bacterial suspension were transferred to 50 ml of LB broth and incubated at 37°C with constant shaking at 300 rpm (Orbital Mixer – Incubator, Ratek Instruments, Boronia, Vic., Australia) until an optical density of 0.3–0.4 (λ=600 nm) was reached. The bacterial cells were then pelleted by centrifugation for 5 min at 4000 rpm (Megafuge1.0, Heraeus). The bacterial pellet was resuspended in 5 ml of TSS (TSS: 10% (w/v) polyethylene glycol, pH 6.5, adjusted with HCl) and, subsequently, 200 µl aliquots were transferred to 1.5 ml microfuge tubes, frozen in liquid nitrogen and stored at –80°C.

Products of the pBin19-pAN7-1 and pPZP200-pAN7-1 ligations were added to Eppendorf tubes containing 200 µl of pre-thawed competent cells and incubated on ice for 20-30 min. The tubes were then placed at 42°C in a water bath for 45 sec and re-transferred to ice for 2 min. After the addition of 800µl of LB broth the tubes were incubated for 90 min at 37°C. The contents of each tube were then transferred to LB agar plates containing the appropriate antibiotic (pBin19-pAN7-1: 50 µg/ml kanamycin; pPZP200-pAN7-1: 50 µg/ml spectinomycin).

To confirm the success of a cloning reaction it was necessary to show the presence of the plasmid within a bacterial colony. Single bacterial colonies were therefore selected and grown in LB broth liquid culture containing the appropriate antibiotic at 37°C for 16 h. Plasmid DNA was then extracted from the bacterial cells using the QIAprep® Spin Miniprep Kit (Qiagen Pty Ltd, Doncaster, VIC, Australia) and digested with informative restriction enzymes. The restriction fragments were separated on 1% agarose/TAE gels, stained with ethidium bromide and visualised under UV light. Bacterial cultures that produced fragments of the expected sizes were isolated for further analysis. Furthermore, liquid cultures of these colonies were converted to glycerol stocks (15% (w/v) glycerol) and stored at -80°C (Sambrook *et al.*, 1989).

2.2.3.3 Transformation of Agrobacterium tumefaciens *cells*

A. tumefaciens strain AGL-0 was obtained from Dr. Angela Gerlich, The University of Adelaide, Discipline of Plant and Pest Science. Competent cells of *A. tumefaciens* were produced using a method similar to the production of competent *E. coli* cells. A single *A. tumefaciens* colony was selected from a YEB-media agar plate (YEB broth; 0.5% (w/v) bacto-tryptone, 0.5% (w/v), yeast extract, 0.1% (w/v), sucrose, 0.5% (w/v), 2 mM $MgSO_4$, pH 7.2: YEB Agar; 1.5% (w/v) agar dissolved in YEB broth) and incubated in 4 ml of YEB broth supplemented with 25 µg/ml rifampicin for 16 h at 28°C with constant shaking at 300 rpm (Orbital Mixer – Incubator, Ratek Instruments). Subsequently, 1 ml of the bacterial suspension was transferred to 100 ml of YEB broth (25 µg/ml rifampicin) and incubated at 28°C with constant shaking at 300 rpm for 2 h (Orbital Mixer – Incubator, Ratek Instruments). The bacterial culture was then centrifuged at 4000 rpm for 10 min (Megafuge1.0, Heraeus). The resulting bacterial pellet was resuspended in 5 ml of TSS (TSS: 10% (w/v), polyethylene glycol pH 6.5 - adjusted with HCl) and, subsequently, 200 µl aliquots were transferred to 1.5-ml Eppendorf tubes, frozen in liquid nitrogen and stored at –80°C.

To Eppendorf tubes containing 200 µl of pre-thawed competent cells, 2 µg of the binary vectors pBin19-pAN7-1 and pPZP200-pAN7-1, respectively, were added. After transferring to liquid nitrogen for 1 min they were placed for 6 min into a water bath at 37°C. Then, 800 µl of LB broth was added and the tubes were incubated for 2 h at 28°C. The contents of each tube were then transferred to YEB agar plates (25 µg/ml rifampicin) containing the appropriate antibiotic (pBin19-pAN7-1, 50 µg/ml kanamycin; pPZP200-pAN7-1, 50 µg/ml spectinomycin).

2.2.3.4 Transformation of R. secalis *Spores*

Fungal spores were harvested from 10-d old fungal cultures grown on Lima bean agar. Five to 10 ml of water was added to a culture and the mycelial mat was gently rubbed with the end of a sterile 10-ml glass pipette. The spore suspension was then decanted into a sterile tube and centrifuged at 5000 rpm for 5 min (Megafuge1.0, Heraeus). Following centrifugation, the supernatant was

discarded and the pellet resuspended in 5 ml of water. The spore suspension was then purified by filtration though sterile glass wool. The titre of the spore suspension was adjusted to $1x10^6$ spores/ml after counting a small aliquot of the purified spore suspension using a Neubauer counting chamber of 0.1 mm (Hausser Scientific, Horsham, PA, USA). The protocol used for ATMT was as previously described by de Groot *et al.* (1998). In brief, a 2-d culture of *A. tumefaciens* containing the appropriate binary vector was grown at 28°C with constant shaking at 300 rpm (Orbital Mixer – Incubator, Ratek Instruments) in minimal media (11.77 mM K_2HPO_4, 10.66 mM KH_2PO_4, 2.57 mM NaCl, 2.03 mM $MgSO_4$, 4.56 mM $CaCl_2$, 8.99 µM $FeSO_4$, 1.51 mM $(NH_4)_2SO_4$, Glucose 11.1 mM) under antibiotic selection corresponding to the binary vector. The optical density of the culture was measured and the necessary dilution made with induction media (composed of minimal media salts with the addition of 40 mM MES, 10 mM glucose, 0.5% (w/v) glycerol, 200 µM acetosyringone, pH 7.5) to reach an OD_{660}=0.15. The dilution was returned to the orbital mixture and incubated under the same conditions for a further 6 h. Spore and *A. tumefaciens* solutions were mixed at a ratio of 1:1 and 200 µl aliquots transferred to induction media plates (induction media supplemented with an additional 5 mM glucose) with a 45 µm nitrocellulose filter placed on the surface. Following a 2-d incubation at 28°C, nitrocellulose filters were transferred to Lima bean agar supplemented with 200 mM cefotaxime and 100 µg/ml hygromycin and incubated under normal fungal growth conditions. ATMT mutants were not single-spored. This was deemed unnecessary because transformant colonies were excised from selective plates with very low colony numbers.

2.2.4 Molecular Analysis

2.2.4.1 DNA Extraction from Fungal Mutants

For the extraction of nucleic acids, 2-wk old mycelial cultures were filtered through Whatman filter paper grade 1 (Whatman International Ltd., Singapore) to separate the liquid media from the fungal material. Fungal mycelia were then frozen in liquid nitrogen and ground in the presence of acid-washed sand (BDH chemicals, Poole, UK) using a mortar and pestle until a fine powder was produced. This powder was then used directly for nucleic acid extraction, or stored at –80°C until required.

To 200 mg of ground fungal mycelia, 700 µl of DNA extraction buffer (1% sarkosyl, 100mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 2% polyvinylpolypyrrolidone, pH 8.5) were added and

mixed constantly for 1 min. The homogenate was then combined with 700 µl of a mixture of phenol/chloroform/isoamyl alcohol (25:24:1) and mixed constantly on an orbital shaker for 15 min. The mixture was then transferred to a 10-ml Serum Gel (Clotted Blood Container) tube (Sarstedt Australia Pty, Ltd., Ingle Farm, SA, Australia) and the upper aqueous phase, separated by the gel matrix of the Serum Gel tube, recovered after centrifugation for 10 min at 6000 rpm (Megafuge1.0, Heraeus). The DNA was precipitated from the supernatant by the addition of 600 µl (0.7 volumes) of isopropanol and 60 µl of 3 M sodium acetate (pH 5.2) and centrifugation at 13000 rpm for 10 min. The DNA pellet was washed once in 70% ethanol, air-dried at room temperature and resuspended in 50 µl of nanopure water.

DNA concentration was estimated by visual comparison of staining intensity with ethidium bromide against standard DNA markers of known concentration on a 1% agarose/TAE gel. Alternatively, estimation was made by measuring the optical density at 260 and 280 nm in a spectrophotometer (Metertech, Inc., Taipei, Taiwan).

2.2.4.2 Probe Preparation for Southern Hybridisation

Three probes were used for Southern analysis of this experimental work. Probe 1 (Fig 2.2), used for detecting the hygromycin resistance gene in plasmid pAN7-1, was a 2072 bp fragment amplified from pAN7-1 vector template using the polymerase chain reaction (PCR). Ms. Annette Boettcher, Discipline of Plant and Pest Sciences, The University of Adelaide, generously provided the PCR oligonucleotide primers (hphNotIfwd and hphNotIrev, see Appendix – Oligonucleotide Primers). Amplification was conducted using a Perkin Elmer GeneAmp® PCR System 9700 (Applied Biosystems, Scoresby, VIC, Australia) in combination with the Xpand high Fidelity Polymerase enzyme (Roche Diagnostics Australia Pty., Ltd., Castle Hill, NSW, Australia) and the following PCR program:

95°C, 3 min.

94°C, 45 seconds. }
60°C, 30 seconds. } 30x
72°C, 2 min. }

72°C, 7 min

To produce probes 2 and 3, (Fig 2.4) two separate restriction digests were performed. Probe 2 was a 3987 bp fragment released from pAN7-1 upon digestion with the restriction enzymes was *Bgl*II and *Hind*III (New England Biolabs, USA). Probe 3 was a 3989bp fragment isolated from pPZP200-pAN7-1 using the restriction enzymes *Kpn*I and *Hind*III (New England Biolabs, USA). All probe fragments were separated on 1% agarose/TAE gels, stained with ethidum bromide, visualised under UV light, excised with a sterile scalpel blade and gel purified using the QIAquick® Gel Extraction Kit (Qiagen Pty Ltd, Australia). Probes 2 and 3 detect the hygromycin resistance gene and segments of their respective binary vectors.

Probes were radioactively labelled using the Random Primed DNA Labelling Kit (Roche Diagnostics Australia Pty Ltd., Australia) according to manufacturer's instructions. [α-^{32}P]-dCTP was obtained from Geneworks (Geneworks Pty Ltd., Hindmarsh, SA, Australia). Following the labelling reaction, the reaction mixture was purified using the Ultraclean™ PCR Clean-up DNA Purification Kit (MO-BIO Laboratories, Inc., West Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Finally, the radioactively labelled probes were denatured by placing them in boiling water for 5 min followed by 5 min on ice.

2.2.4.3 Southern Hybridisation

The following is an adaptation of the original protocol published by Southern (1975). Approximately 10 µg of total genomic fungal DNA were digested with the required restriction enzyme. DNA fragments were separated on 1.0% agarose/TAE gels for 6-8 hours at 100 V. DNA was denatured by immersion and agitation of the gels in a solution of 1.5 M NaCl, 0.5 M NaOH for 20 min. Then, gels were washed in 10xSSC (1.5 M NaCl, 0.3 M tri-sodium citrate) for 2 min. Each gel was inverted and placed upon the transfer apparatus consisting of a pad of two sheets of 3 mm Whatman paper (Whatman International Ltd., Singapore) layered on a further two sheets of 3 mm Whatman paper with the ends of the underlying two sheets immersed in a reservoir of 10xSSC. A Hybond N membrane (Amersham Biosciences, Piscataway, NJ, USA) was laid upon the gel and marked with a pencil to indicate the location of gel features. Two sheets of 3 mm Whatman paper were placed upon the membrane followed by an absorbent stack. DNA was transferred to the membrane by capillary action for 8-12 h overnight using 10xSSC as the transfer buffer. After the transfer apparatus was disassembled the membrane was dried with 3 mm Whatman paper and the

DNA fixed to the membrane by placing it onto a transilluminator for exposure to UV light for 4 min. Membranes were then stored at 4°C until required.

Hybridisation bottles (Bartelt Instruments, Heidelburg, VIC, Australia) were cleaned and pre-warmed at 65°C. Once dry, 25 ml of hybridisation solution (1 M NaCl, 1% SDS, 10% dextran sulphate) and 1 ml (5 mg/ml, autoclaved) salmon sperm DNA (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia) pre-boiled for 5 min were added to the bottle, mixed and pre-warmed in a 65°C oven. Each membrane was then rolled into a cylinder and placed inside the bottle. Bottles containing unrolled membranes were placed into a 65°C hybridization oven (Ratek Instruments Pty., Ltd., Boronia, VIC, Australia) and rotated for 4 h. Then, the solution containing the respective labelled probe (see 2.2.4.2) was poured into the hybridisation bottle containing the pre-hybridised membranes. Hybridisation bottles were returned to the rotor and hybridisation performed overnight at 65°C.

Residual unbound probe was removed by washing the membrane with increasing stringency at 65°C for 25 min intervals with gentle shaking using the following SSC solutions: 2xSSC, 0.1% (w/v) SDS (sodium dodecyl sulphate); 1xSSC, 0.1% (w/v) SDS; 0.5xSSC, 0.1% (w/v) SDS; 0.2xSSC, 0.1% (w/v) SDS. Following completion of the wash cycles, membranes were blotted dry, sealed in plastic bags and exposed to X-ray film for 1-14 d depending on signal intensity. X-ray film was developed using an AGFA CP1000 processor (Agfa-Gevaert N.V., Mortsel, Belgium). If the membranes were to be reused, they were placed in boiling stripping solution (0.1% (w/v) SDS, 2mM Na_2EDTA, pH 8.0) for approximately 30 min to remove the radioactive probe after which the membrane was dried and immediately reused.

2.3 Results

2.3.1 Mutagenesis of *R. secalis* by REMI

2.3.1.1 Comparison of Restriction Enzymes and Fungal Strain Using Circular Plasmid

To identify the combination of fungal strain, circular pAN7-1 vector and restriction enzyme that would provide the most efficient transformation, experiments were performed using two restriction enzymes, *Bam*HI and *Hind*III (20 units each) and the two fungal strains 5 and H2.5. The

transformation efficiencies obtained with these combinations of strains and enzymes are presented in Table 2.1.

Table 2.1: Transformation efficiencies* obtained with two fungal strains and two restriction enzymes.

		Transformation Efficiencies*	
Strain	Experiment	*Bam*HI^	*Hind*III^
H2.5	(i)	7.7×10^{-7}	4.13×10^{-7}
5	(i)	1.56×10^{-5}	3.78×10^{-6}
5	(ii)	7.48×10^{-6}	6.44×10^{-6}
5	(iii)	1.45×10^{-5}	1.24×10^{-5}
5	(iv)	1.54×10^{-5}	n.d.$^{\Omega}$

*The terms "transformation efficiency" and "transformation frequency" are used synonymously in the literature to describe the number of transformed cells per mass of plasmid DNA used for transformation (transformants/µg DNA). However, throughout this dissertation the term "transformation efficiency" is used to describe the proportion of transformed cells per wild type cell (fungal protoplast or spore cell) subjected to transformation.

^ 20 units of the *Bam*HI and *Hind*III restriction enzymes were added to the transformations.

$^{\Omega}$ n.d. – no data recorded

It is apparent that strain 5 gave higher transformation efficiency than strain H2.5 for each enzyme (Table 2.1). Fungal strain 5 was used in three transformations in the presence of *Hind*III and four transformations with *Bam*HI added. Strain 5 transformation efficiencies tended to be generally higher with *Bam*HI (7.5-15.6 x 10^{-6}) than with *Hind*III (3.8-12.4 x 10^{-6}). This trend also appears to be apparent in strain H2.5 where *Bam*HI also gave higher transformation efficiency than *Hind*III.

2.3.1.2 The Effect of Restriction Enzyme Concentration on Transformation Efficiency

R. secalis strain 5 was used to examine the effect of restriction enzyme concentration on transformation efficiency. Due to logistical limitations, only one restriction enzyme, *Bam*HI, was

used in 10 separate transformation experiments. *Bam*HI was selected because this enzyme gave higher transformation efficiencies in the earlier experiments performed during the current study (see 2.3.1.1). Five different enzyme concentrations were combined with both the pre-linearised and the circular form of the plasmid pAN7-1. The resulting transformation efficiencies are presented in see Table 2.2.

Table 2.2: The effect of plasmid form and *Bam*HI concentration on transformation efficiency.

Plasmid Form	Enzyme Amount (units)	Transformants Generated	Transformation Efficiency
Circular	0	229	$1.17x10^{-5}$
Circular	20	433	$2.22x10^{-5}$
Circular	50	387	$1.99x10^{-5}$
Circular	100	539	$2.76x10^{-5}$
Circular	200	224	$1.15x10^{-5}$
Linear	0	n.d.*	n.d.*
Linear	20	86	$4x10^{-6}$
Linear	50	53	$2.47x10^{-6}$
Linear	100	39	$1.81 x10^{-6}$
Linear	200	28	$1.30x10^{-6}$

*n.d. the "no-enzyme" control became contaminated during the experiment making accurate scoring impossible.

Several interesting points were raised by the results of this experiment. Firstly, at each enzyme concentration the circular form of the plasmid was more efficient than the linearised form. Secondly, the addition of a restriction enzyme produced an approximately two-fold increase in the transformation efficiency for the circular plasmid. This was observed with all enzyme concentrations except with the highest concentration of 200 units that shows the same transformation efficiency as the control in the absence of *Bam*HI. Finally, when the circular form of the plasmid was used transformation efficiency was not found to be a function of enzyme concentration. In contrast, increasing enzyme concentrations appeared to decrease transformation efficiency when the linear form of the plasmid was used.

2.3.1.3 Copy Number and Integration Pattern

Single-site single-copy integrations provide the most easily analysable mutants in tagging studies. Hence, there was interest in the fraction of mutants containing single-copy insertions produced by REMI. Consequently, 15 mutants selected at random from REMI transformations that included 20 units of the restriction enzyme *Bam*HI and circular plasmid were analysed for copy number and integration pattern with specific emphasis on determining the number of single copy mutants within this sample. The enzyme concentration of 20 units was selected because it represents the lowest concentration yielding a two-fold increase in transformation efficiency as compared to the control in the absence of enzyme.

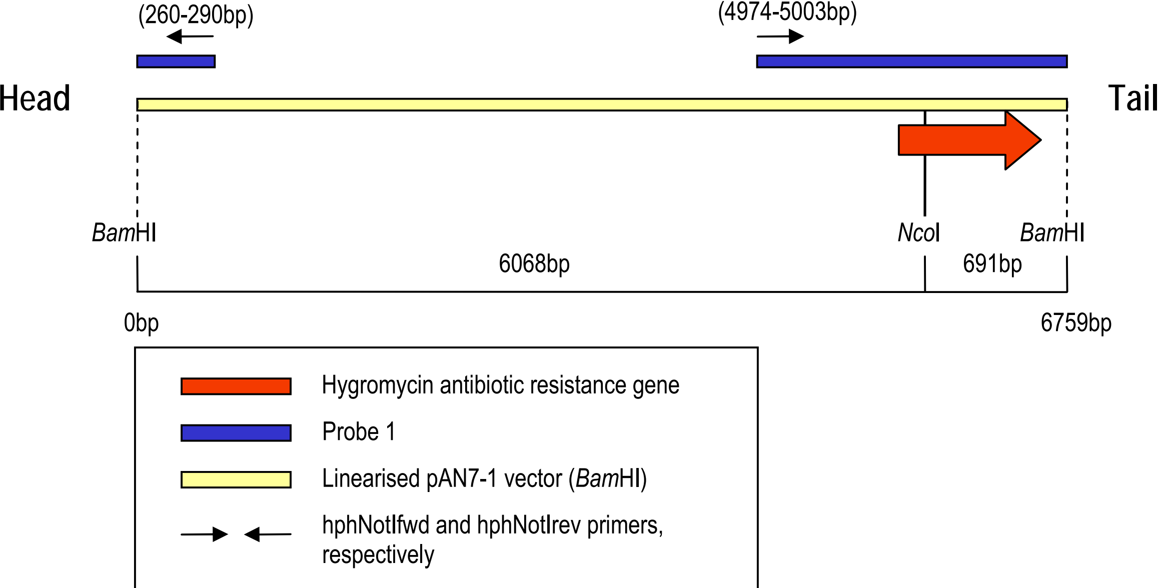


Figure 2.2: Location of probe and restriction sites used to analyse the integration type in the REMI mutants (Note: The diagram shows plasmid pAN7-1 linearised with *Bam*HI. This representation is based on the assumption that the vector was linearised by the restriction enzyme during the transformation process). *Nco*I cleaves the plasmid pAN7-1 within the sequence of probe 1.

Two restriction enzymes were selected to determine the number of single copy integrations present in the sample of 15 mutants, *Eco*RV and *Nco*I. *Eco*RV does not cut within the pAN7-1 sequence and, therefore, yields the number of different sites at which integrations have

occurred in the fungal genome. Furthermore, because there is no *Eco*RV restriction site in the pAN7-1 sequence each band present on the autoradiograph (Fig 2.3) should exceed the length of the pAN7-1 vector (6759 bp).

The second informative enzyme, *Nco*I, has a restriction site in the pAN7-1 sequence within the region hybridising with probe 1 (Fig 2.2). This digest is useful to identify tandem integrations present at a single integration site. If more than one copy of the pAN7-1 plasmid integrated into the same genomic location, adjacent molecules would have three possible orientations, head-to-head, head-to-tail or tail-to-tail, or combinations thereof. Where tandem integrations are present, a minimum of three bands should be visible on the autoradiograph. If one copy of pAN7-1 has integrated at a single locus within the fungal genome, an *Nco*I digest combined with probe 1 would show two fragments larger than 6068 bp and 691 bp. (Figure 2.3).

Southern analysis of the 15 REMI mutants showed that 6 of the 15 mutants contained clear single-copy integrations. These mutants, R4, R5, R7, R8, R14 and R15 all show the expected fragment size for both restriction enzymes, *Eco*RV and *Nco*I. The remaining 9 mutants either have more complex integration patterns, or the banding displayed for these mutants on the autoradiograph have been complicated by partial rather than complete enzymatic digestion. However, partial digestion was considered unlikely because all digests were similar when the Southern gel was visualised under UV light (data not shown). Several mutants have banding patterns consistent with complex integrations. Mutants R9 and R13 produce two bands after *Eco*RV digestion indicating that two pAN7-1 integrations occurred at different sites in the fungal genome. This data is supported by the *Nco*I digest that produced four bands with sizes consistent to two integrations at different locations in the genome. Two mutants, R2, and R6, show banding patterns consistent with tandem integrations in an tail-to-tail orientation producing fragments that appear to be 1382 bp in length (Fig 2.2). The remaining mutants have unexplained banding patterns. However, a detailed interpretation of these mutants was not made because of time constraints, and the primary focus of this section of the study was to determine the approximate frequency of single-copy integration events that occur using REMI in *R. secalis*.

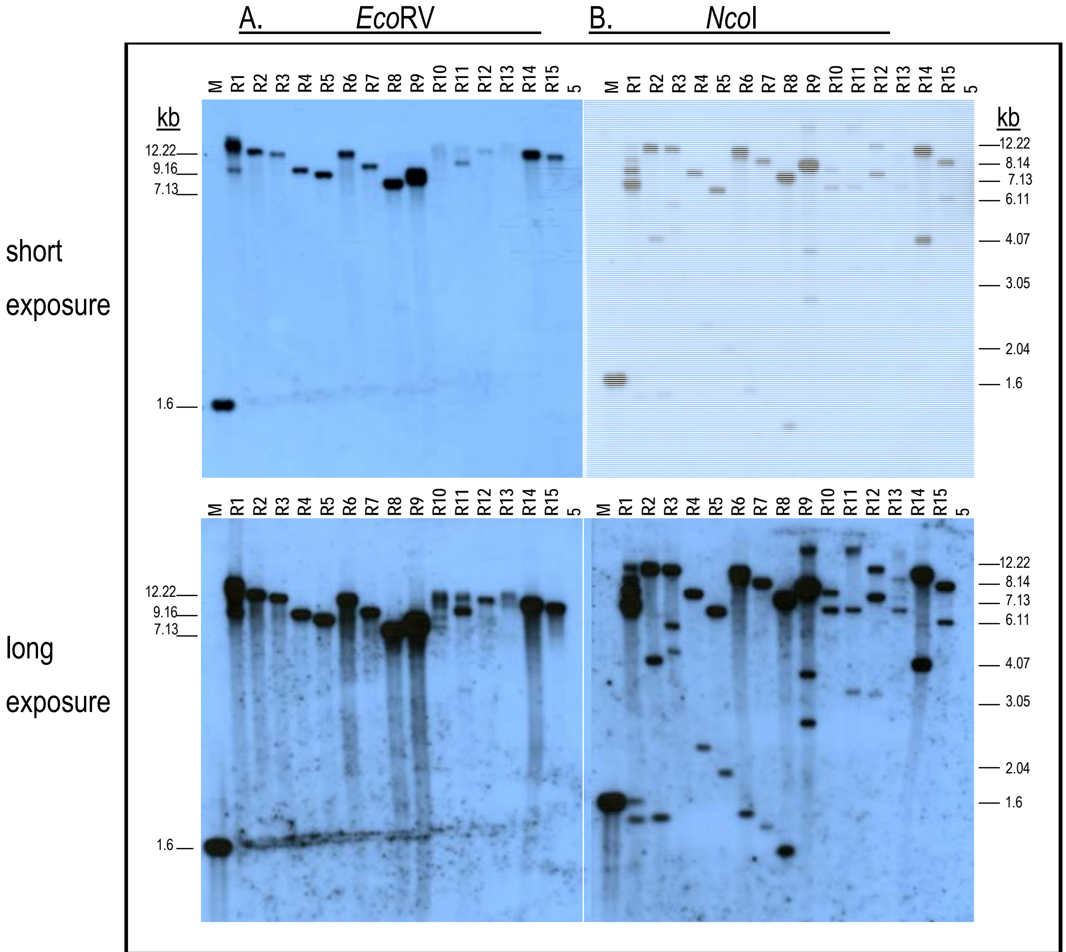


Fig 2.3: Southern hybridisation of DNA from REMI mutants digested with the restriction enzymes A. *Eco*RV and B. *Nco*I with short (top) and long (bottom) exposure time. Unequal loading caused variable band intensity. DNA abbreviations: M – marker; R1-R15 – DNA from independent transformants; 5 – DNA from wild type strain 5.

2.3.2 Mutagenesis of *R. secalis* by ATMT

A. tumefaciens had not previously been used for the transformation of *R. secalis*, representing an opportunity to examine transformation efficiency, integration stability, copy number and integration pattern in the fungus. Two binary vectors were used to transform strain UK7 of *R. secalis*. The transformation was considered successful and all experimental controls produced the expected results. However, one experimental anomaly occurred during the transformation process. Following co-cultivation of fungal conidia and *A. tumefaciens* cells, the cellular mix was plated onto induction media with and without an acetosyringone supplement. The transformation performed

with the binary vector pBin19+pAN7-1 produced no hygromycin transformants after the 2-d incubation on induction media in the absence of acetosyringone. In contrast, transformations using the binary vector pPZP200+pAN7-1 yielded six hygromycin transformants after 2-d incubation under the same conditions. This will be discussed later. The following results were all obtained in the presence of acetosyringone.

2.3.2.1 Transformation Efficiency and Binary vectors

ATMT transformation efficiency was examined and compared with transformation efficiencies from other filamentous fungi and with REMI transformation efficiency.

Table 2.3: ATMT efficiencies of different fungal species with different binary vectors.

Binary Vector	Fungal Species	Transformation Efficiency	Reference
pBin19 + pAN7-1[a]	*Rhynchosporium secalis*	1.1×10^{-3}	-
pPZP200 + pAN7-1	*Rhynchosporium secalis*	3.4×10^{-4}	-
REMI[b]	*Rhynchosporium secalis*	1.5×10^{-5}	(section 2.3.1)
pUR5750	*Aspergillus awamori*	1×10^{-4} – 9×10^{-4}	de Groot *et al.*, 1998
pUR5750	*Aspergillus niger*	5×10^{-7}	de Groot *et al.*, 1998
pUR5750	*Colletotrichum gloeosporioides*	1.3×10^{-4} – 5×10^{-5}	de Groot *et al.*, 1998
pUR5750	*Fusarium venenatum*	2.5×10^{-6}	de Groot *et al.*, 1998
pUR5750	*Trichoderma reesei*	1.2×10^{-4} – 2.4×10^{-5}	de Groot *et al.*, 1998
pUR5750	*Neurospora crassa*	5×10^{-4}	de Groot *et al.*, 1998
pUR5750	*Agaricus bisporus*	1×10^{-7} – 5×10^{-7}	de Groot *et al.*, 1998
pUR5750	*Aspergillus giganteus*	5×10^{-6} – 7.9×10^{-5}	Meyer *et al.*, 2003
pBHt1	*Fusarium oxysporum*	3×10^{-4} – 5×10^{-4}	Mullins *et al.*, 2001
pBHt2	*Magnaporthe grisea*	$>1 \times 10^{-3}$	Rho *et al.*, 2001

[a] pBin19+pAN7-1 is the same vector as pUR5750 but constructed independently.

[b] REMI efficiency was calculated by averaging five independent strain 5 transformations using 20 units *Bam*HI and circular pAN7-1 plasmid (section 2.3.1, Tables 2.1 and 2.2).

According to the results presented in Table 2.3, ATMT of *R. secalis* was highly efficient compared to transformations of other fungal species with the exception of *M. grisea*. The binary vector pBin19+pAN7-1 gave a higher efficiency than pUR5750 in any other fungal species. In addition, the pBin19 construct was approximately three times more efficient than the pPZP200 construct at transferring the hygromycin antibiotic resistance gene to *R. secalis* conidia.

2.3.2.2 Number of Integration Sites, Integration Pattern and Stability

To determine the number of integration sites, the integration pattern and the stability of integrations obtained by ATMT, Southern hybridisation was performed. To assess the mitotic stability of the T-DNA integrations, mutants included in the Southern hybridisation passed through six generations after transformation without antibiotic selection. Furthermore, the Southern analysis was extended to include informative restriction enzymes to elucidate the T-DNA copy number and integration pattern. Two different probes, probe 2 and probe 3, and different restriction enzyme combinations were used depending on the binary vector generating the mutant. The location of the probes with respect to T-DNA structure is presented in Fig 2.4.

The T-DNA region, when present in a binary vector, is delimited by two border sequences. The repetitive left border (LB) and right border (RB) elements are the site of specific endonucleolytic cleavage that generates a single-stranded molecule that is ultimately transferred to the target genome (Stachel *et al.*, 1987). However, cleavage within the border sequences has been reported as inconsistent and consequently slight variations in T-DNA size will occur (Rho *et al.*, 2001; Mullins *et al.*, 2001; Krizkova and Hrouda, 1998; Kumar and Fladung, 2002). For practicality, the following section discusses size fragments assuming the T-DNA fragment contains intact RB and LB sequences, even though this is not always the case.

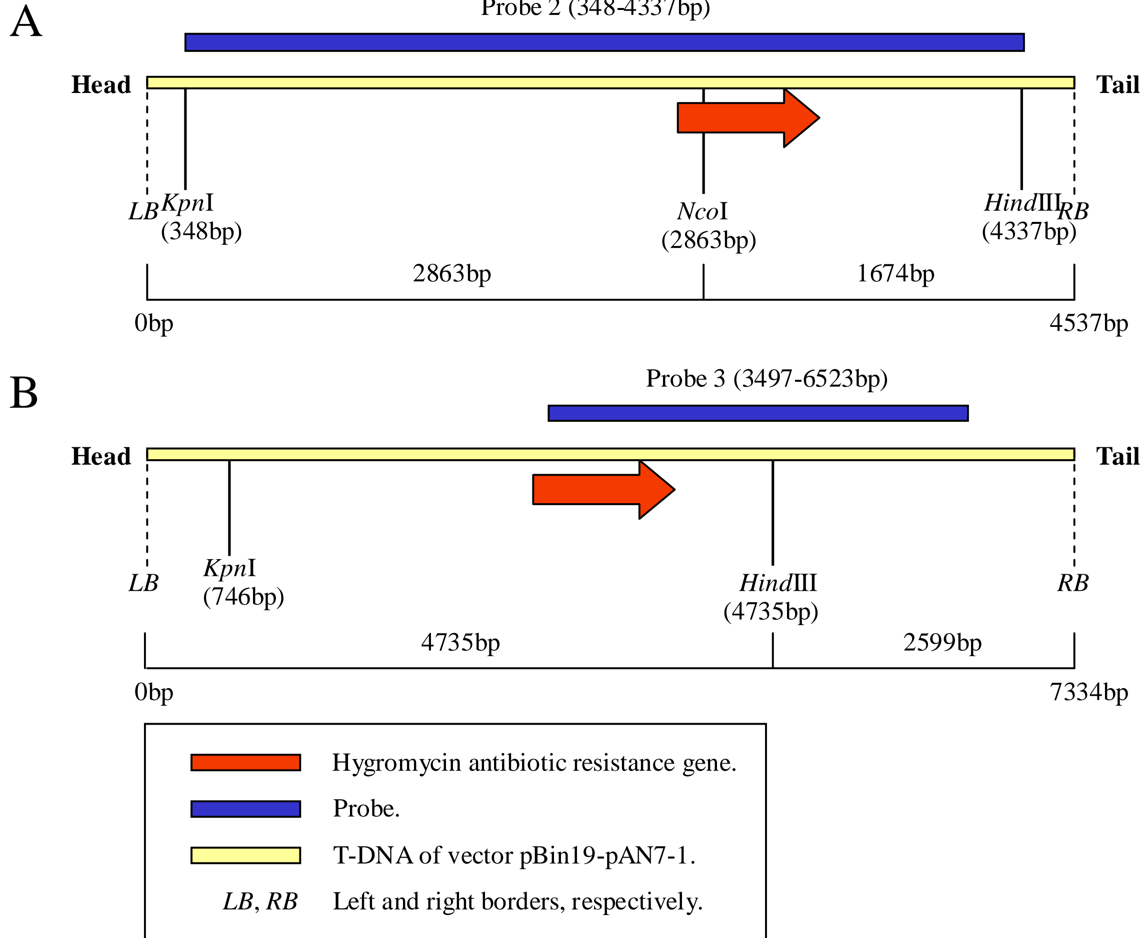


Figure 2.4: Location of probe and restriction sites used to analyse stability of integration and integration type in the ATMT mutants. A. T-DNA structure of pPZP200+pAN7-1. B. T-DNA structure of pBin19+pAN7-1.

From transformations using binary vectors pPZP200+pAN7-1 and pBin19+pAN7-1, 15 mutants each were selected at random. Two restriction enzymes were used to digest the DNA of each individual transformant. Restriction enzyme selection was based on their ability to identify mutants that contained single site-single copy T-DNA integrations. Autoradiographs of Southern blots from mutants transformed with the binary vectors pPZP200+pAN7-1 and pBin19+pAN7-1 are presented in Fig 2.5 and 2.6, respectively.

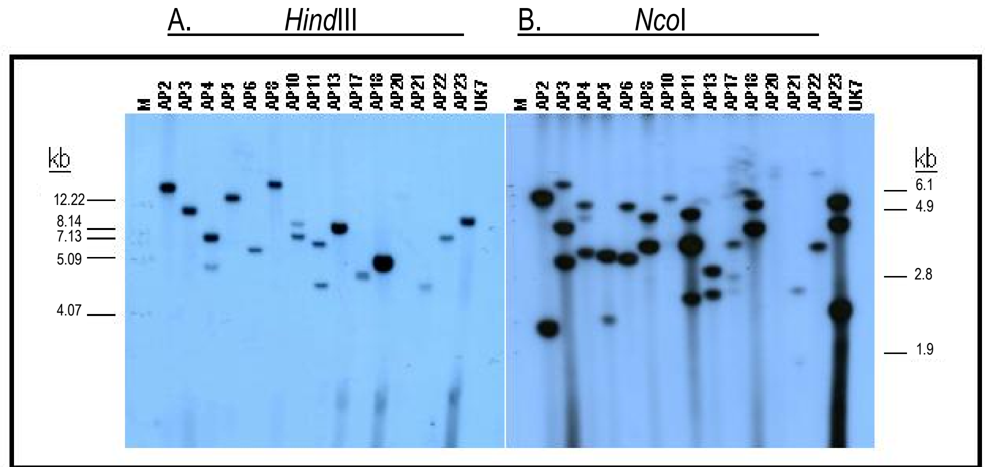


Figure 2.5: Southern analysis using probe 2 of genomic DNA from ATMT mutants transformed with vector pPZP200+pAN7-1. Digests were performed with the restriction enzymes *Hind*III and *Nco*I. DNA abbreviations: M – marker; AP2-AP23 – DNA from independent transformants; UK7 – DNA from wild type strain UK7.

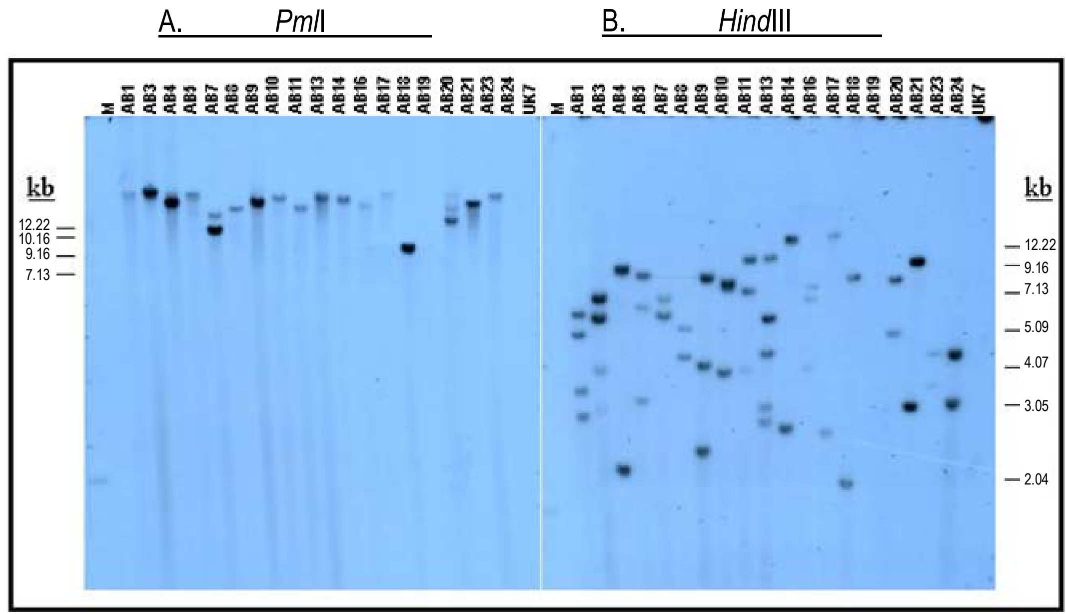


Figure 2.6: Southern analysis using probe 3 of genomic DNA from ATMT mutants transformed with vector pBin19+pAN7-1. Digests were performed with the restriction enzymes *Pml*I and *Hind*III. DNA abbreviations: M – marker; AB1-AB24 – DNA from independent transformants; UK7 – DNA from wild type strain UK7.

Probes 2 and 3 showed no cross hybridisation with the DNA from the wild type parental strain UK7 and therefore specifically hybridise to the T-DNA of their respective binary vectors, pPZP200+pAN7-1 (Fig 2.5) and pBin19+pAN7-1 (Fig 2.6). In contrast, DNA from all mutants except two showed the presence of the T-DNA indicating that integration remained mitotically stable in the fungal genome after six generations. In the case of mutant AP20 and AB19, no digested genomic DNA was visible on the gel (data not shown) thus explaining these negative results.

To determine the number of single-copy integrations in mutants transformed with the binary vector pPZP200+pAN7-1 the restriction enzymes *Hind*III and *Nco*I were selected. Both enzymes cut the T-DNA, however, *Nco*I digests the T-DNA within the region to which probe 2 hybridises, whereas a *Hind*III site is located in the T-DNA directly adjacent to this region (Fig 2.4A). A combined interpretation of the Southern blots (Fig 2.5) allowed a determination of the number of single-copy integrations present in the pool of 15 mutants. If the autoradiograph showed a single band larger than 4336 bp after *Hind*III digestion, it could be concluded that T-DNA integration occurred at only one site in the fungal genome unless a band consistent with 8672 bp in length was present indicating the possibility of a head-to-head T-DNA integration. Tandem integrations were excluded in this case by interpretation of the Southern results of *Nco*I digests. If two bands larger than 2862 bp and 1673 bp were produced by *Nco*I digestion of DNA from the same mutant that produced a single band after *Hind*III digestion, it was concluded that this mutant contained one copy of the T-DNA integrated at a single site in the fungal genome. Single integrations were observed in 8 of the 15 mutants, AP2, AP5, AP6, AP8, AP13, AP18, AP21 and AP22. Mutant AP23 shows a banding pattern consistent with a head-to-head integration. Head-to-head integrations produce one single band 8672 bp in length upon digestion with *Hind*III and three fragments upon *Nco*I digestion, one of 5724 bp and two >1673 bp. The remaining five mutants AP3, AP4, AP10, AP11 and AP17 are assumed to have more complex integration patterns or may be the result of incomplete restriction enzyme digestion.

The number of single-copy integrations was also determined in mutants transformed using the binary vector pBin19+pAN7-1. The restriction enzymes *Pml*I and *Hind*III were used to digest DNA from 19 mutants of this transformant pool. *Pml*I does not digest the T-DNA sequence of pBin19+pAN7-1 and, therefore, the number of bands observed on the autoradiograph from each

mutant reflects the number of integration sites. A second restriction digest with the enzyme *Hind*III, that cuts the T-DNA once within the region to which probe 3 hybridises, then determined if a single copy is located at the single integration site or whether multiple copies exist at one location in tandem orientation. If one copy of the T-DNA integration inserted into the fungal genome at a single site, the expected fragment sizes for the digests are: one band larger than 7333 bp for *Pml*I and two bands larger than 2362 bp and 4971 bp for *Hind*III. In total, seven mutants, AB4, AB8, AB10, AB14, AB17, AB21 and AB23 produced banding patterns that were consistent with the sizes expected for simple, single-copy integrations. Mutant AB18 also produced a banding pattern consistent with a single-copy single-site integration (Fig 2.6). However, in this case one of the band sizes was approximately 2kb in length, smaller than the expected 2.3 kb fragment that should be observed upon *Hind*III digestion. The mutant AB16 shows banding sizes consistent with a head-to-tail tandem integration (Fig 2.4B). Expected band sizes for this type of integration are one fragment larger than 14866 bp when digested with *Pml*I and three fragments, one 7433 bp and two larger than 4971 bp and 2462 bp, when digested with *Hind*III. The remaining seven mutants did not produce results that could be easily interpreted. These banding patterns are assumed to result from either partial digestion or a more complex integration pattern.

To summarise, both binary vectors produced mutants with T-DNA integrations that were stable over a period of 6 generations without antibiotic selection. Furthermore, with vectors pPZP200-pAN7-1 and pBin19-pAN7-1 greater than 50% and 40%, respectively, of single-copy T-DNA integrations were obtained.

2.4 Discussion

At the inception of this study, the only method for transforming *R. secalis* was the polyethylene glycol/$CaCl_2$ transformation protocol described by Rohe *et al.* (1995) and its derivative for REMI transformation. However, the existence of ATMT protocols that had been established for other filamentous fungi (de Groot *et al.*, 1998; Mullins *et al.*, 2001; Meyer *et al.*, 2003; Rho *et al.*, 2001) provided the opportunity to adapt these protocols to *R. secalis*. In this study, the ATMT method

was successfully adapted to *R. secalis* using two different binary vectors, one that had already been used to transform filamentous fungi and one that was used here for the first time.

2.4.1 REMI Transformation

The addition of a restriction enzyme during transformation has been reported to have a number of beneficial effects to the transformation of different fungal organisms (reviewed in Maier and Schafer, 1999). In *R. secalis*, REMI of circular pAN7-1 plasmid in the presence of *Bam*HI increased transformation efficiency at all enzyme levels tested with the exception of the highest enzyme level. This increase in transformation efficiency is consistent with a number of different fungal species including *Cochliobolus heterostrophus* (Lu *et al.*, 1994), *Penicillium paxilli* (Itoh and Scott, 1997), *Coprinus cinereus* (Cummings *et al.*, 1999), *Magnaporthe grisea* (Shi *et al.*, 1995), *Mycosphaerella zeae-maydis* (Yun *et al.*, 1998) and *Ustilago maydis* (Bolker *et al.*, 1995).

The REMI transformation experiments performed in this study showed that strain 5 of *R. secalis* in combination with the restriction enzyme *Bam*HI yielded the highest transformation efficiencies (Table 2.1). Furthermore, it was demonstrated that transformation efficiency varied substantially between REMI experiments, a finding consistent with previous REMI transformations performed with *R. secalis* (data not shown). The causes for the observed variations in transformation efficiency remain unclear and, where possible, transformations were analysed independently of one another.

The use of a restriction enzyme in combination with circular pAN7-1 plasmid increased transformation efficiency at all enzyme concentrations except for the highest *Bam*HI concentration. High *Bam*HI concentrations were also inhibitory in transformation studies in *M. grisea* (Shi *et al*., 1995). However, the latter studies were performed using the vector pAN7-2 linearised prior to the transformation process. The reasons for the inhibitory effect of high *Bam*HI concentrations on the transformation process are as yet unclear. It is possible that restriction enzymes are responsible for damaging the fungal genome (Sweigard *et al.*, 1998). However, Shi *et al.* (1995) observed no decrease in protoplast viability at the highest enzyme concentrations and, therefore, could not correlate the reduction of transformation efficiency to a loss of viable protoplasts.

Itoh and Scott (1997) suggested that the rate-limiting step for plasmid integration is the presence of target sites available for the integration of foreign DNA, rather than the availability of the foreign DNA molecule. Following this logic, it would be considered likely that REMI transformations that utilise between 20 and 100 units of *Bam*HI are able to provide enough target sites to satisfy the amount of circular plasmid (10 µg) used in the transformation.

In the majority of reported REMI studies linearised plasmid DNA was used rather than a circularised plasmid. However, Linnemannstons *et al.* (1999) compared both forms of pAN7-1 in REMI transformations of *Gibberella fujikuroi*. Both enzymes used, *Xba*I and *Hind*III, yielded either comparable or higher transformation efficiencies with circular pAN7-1 plasmid than with linearised plasmid. These data are in agreement with the results obtained by transforming *R. secalis* with linearised and circular plasmid DNA in the presence of *Bam*HI. When circular plasmid was transferred to fungal protoplasts in the presence of a restriction enzyme, *R. secalis* showed an approximately two-fold increase in transformation efficiency compared to the transformation efficiency in the absence of a restriction enzyme (Table 2.2).

The REMI transformation utilising the lowest amount of the restriction enzyme *Bam*HI and circular plasmid was examined to determine the number of single copy integrations. Only 6 out of 15 mutants (40%) exhibited clear simple integrations of plasmid DNA at a single site in the fungal genome. The remaining 9 mutants displayed more complex integration patterns that would require more detailed analysis to unravel the integration process. This was avoided in the current study due to concern about the time and benefit of analyses required to further study these apparently more complex integration events.

2.4.2 ATMT

This study represents the first report on the successful ATMT of the fungal pathogen *R. secalis*. In the presence of acetosyringone, transformant colonies were produced at an efficiency of 1.1×10^{-3} for plasmid pBin19+pAN7-1 and 3.4×10^{-4} for plasmid pPZP200+pAN7-1. In all cases the expected results were obtained with the exception of six hygromycin resistant colonies that grew out of a 2-d co-cultivation with *A. tumefaciens* cells containing the binary vector pPZP200+pAN7-1 on solid induction medium lacking acetosyringone. Acetosyringone is a phenolic compound that enables the activation of virulence genes on the Ti plasmid within *A. tumefaciens* cells that subsequently

mobilise the T-DNA segment from the binary vector to the target genome (Bundock, *et al.*, 1995). This phenolic compound was described as essential for generating insertion mutants of filamentous fungi (de Groot *et al.* 1998; Mullins *et al.*, 2001; Rho *et al.*, 2001; Meyer *et al.*, 2003; Maruthachalam *et al.*, 2008). Hence, in the absence of acetosyringone no transformants should be expected. However, bacterial cells are pre-cultivated for 6 h in liquid induction medium containing acetosyringone and it is this source of the activator that is considered responsible for the six mutants observed.

Two binary vector backbones produced successful transformants. The binary vector pBin19 had been previously used in the transformation of other filamentous fungi. In contrast, this is the first record of the binary vector pPZP200 being used in fungal transformation. The results of the present study indicate that for the transformation of *R. secalis* conidia, the binary vector pBin19 is three times more efficient than the binary vector pPZP200. However, to confirm this difference additional independent repeat experiments comparing the binary vectors would have to be performed to eliminate the possibility of normal variation. The results also show that transformation efficiencies obtained with both binary vectors in *R. secalis* compared to other filamentous fungi appeared equivalent or greater (Table 2.3).

Both binary vectors produce T-DNA integrations that are mitotically stable and the numbers of single-copy integrations produced with both vectors appear roughly comparable. Furthermore, the number of >50% single-copy integrations produced by ATMT of *R. secalis* with binary vector pPZP200-pAN7-1 indicates that this transformation protocol is suitable for future research programs.

One slightly anomalous result was observed during Southern analysis of the AB series of transformants generated with the pBin19 vector (Fig 2.6). DNA from one transformant, AB18, when digested with *Hind*III showed a band smaller than the expected minimum size of 2599 bp (Fig 2.4B). This unexpected result may be explained by T-DNA cleavage and shortening prior to integration, which is one form of processing that occasionally occurs during ATMT. Truncation has also been observed during T-DNA transfer in other filamentous fungi (de Groot *et al.*, 1998), plants (Tinland, 1996) and yeast (Bundock *et al.*, 1995). Hence, the shortened band in mutant AB18 may be a truncated version of the integrated T-DNA that still contains a functional hygromycin antibiotic resistance gene.

Several differences exist between the two binary vectors. pPZP200 is considerably smaller than pBin19 (6741 bp compared to 11777 bp), is a higher copy number vector and has a shorter T-DNA region with an equivalent multiple cloning site (Hajdukiewicz *et al.*, 1994; Frisch *et al.*, 1995). These points indicate that cloning and general handling of pPZP200 is less cumbersome than that of pBin19. Consequently, even though transformation efficiency may be higher using pBin19, it appears reasonable to conduct future transformation experiments preferentially with the binary vector pPZP200.

2.4.3 Comparing ATMT and REMI as Transformation Methods for *R. secalis*

A comparative assessment of the two transformation methods used in this study firstly requires a definition of criteria for proper comparison. Linnemannstons *et al.* (1999) observed that genomic rearrangements, in the form of deletions, occurred in transformations in the presence and absence of restriction enzymes during transformation. However, Sweigard *et al.* (1998) suggest that cutting of the chromosome by restriction enzyme activity leads to imperfect DNA repair and this is why there are a number of untagged mutations in REMI transformations (Sanchez *et al.*, 1998; Balhadere *et al.*, 1999; Epstein *et al.*, 1998). In the current study, REMI gave a high transformation efficiency at the lowest enzyme concentration when circular plasmid was used. Furthermore, if enzyme concentration contributes to untagged mutations caused in the fungal genome by genomic rearrangements such as deletions and translocations, it is logical to assume that low enzyme concentrations represent the enzyme levels that would cause the least damage to the fungal genome. Hence, circular plasmid combined with the lowest *Bam*HI concentration was chosen as the REMI transformation condition that was compared to ATMT.

As previously mentioned, the binary vector pPZP200 would be preferentially selected for any further transformation experiments undertaken with *R. secalis*. Therefore, transformation utilising pPZP200 would be compared to the REMI conditions described above. It should be noted however that the two transformation methods were performed on different fungal strains, REMI on strain 5 and ATMT on strain UK7. If it is assumed that there are no differences between the two strains, using the lowest enzyme concentration for REMI and the binary vector pPZP200 in ATMT, it was demonstrated that REMI yielded lower transformation efficiencies and fewer single-copy

integrations than ATMT. Furthermore, the ATMT protocol is likely to incur less damage to the fungal genome during the transformation process and is less labour-intensive than the REMI protocol. The conclusion drawn from the comparison of the two transformation methods is that ATMT is the favourable method to generate mutants to be used in an insertion mutagenesis study. However, since several hundred REMI mutants were generated first, mutants that are described for the remainder of the thesis were generated by REMI unless other information is provided.

Chapter 3: Identification of Non-Pathogenic Mutants

3.1 Introduction

To identify non-pathogenic mutants in an insertional mutagenesis tagging approach, a phenotypic screening program is required. This program should allow the identification of *R. secalis* mutants that are no longer capable of colonising a host plant. Successful infection of barley by the pathogen causes distinctive scald symptoms (Fig 3.1). However, these symptoms may vary depending on the severity of the infection. Scald lesions occur initially as dark blue-grey areas on the leaf that eventually become necrotic. These necrotic areas develop brown-black borders giving the characteristic scald spots. Disease symptoms appear primarily on the sheaths and blades of leaves. However, in severe cases of natural infections, glumes, awns and embryos may also be affected (Jenkins and Jemmett, 1967; Habgood and Hayes, 1971).

Previous studies vary greatly in the percentage of non-pathogenic mutants obtained by REMI transformations (reviewed by Kahmann and Basse, 1999). The highest percentage of non-pathogenic mutants yielded from REMI mutagenesis is 2.8% in *Ustilago maydis* (R. Kahmann, unpublished). Previous REMI mutagenesis studies conducted with *R. secalis* recorded a similar number of non-pathogenic mutants between 2-3% (S. Albert, unpublished). For the purpose of this study it was assumed that a pool of approximately 500 insertional mutants would be sufficient starting material for the identification of gene(s) involved in pathogenicity. Thus, 453 REMI mutants were generated using the transformation protocols detailed in chapter 2 and screened. In addition to these 453 mutants, 81 mutants from a previous mutagenesis approach (see 3.2.4) were also included in the screening program.

Due to the large number of mutants requiring screening three rounds of screening were employed. In the first two rounds a rapid inoculation protocol was used that was followed by a third, more sensitive and time consuming inoculation procedure.

3.2 Materials and Methods

3.2.1 Plant Growth Conditions

The barley variety Sloop contains no known genes conferring resistance to the scald pathogen and is hence considered universally susceptible. During the course of this project it was used as the sole host variety. Sloop seeds were originally provided by Dr. Steven Jefferies, Department of Plant Science, University of Adelaide. This variety was developed by the University of Adelaide's Barley Breeding Program and can be identified by the accession number WI2875-22. The seed stock was multiplied at Callington in South Australia.

All plants were grown in a potting mix, made up as follows: Forty-five L of composted pine bark potting mix was combined with 90 g of 8-9 month Osmocote (Scotts Australia Pty Ltd, Baulkham Hills, NSW, Australia), 45 g of high phosphorous, 45 g of 3-4 month Osmocote (Scotts Australia Pty Ltd, Baulkham Hills, NSW, Australia), 90 g of IBDU Slow Release Nitrogen (TROPIGRO Pty Ltd, Winnellie, NT, Australia), 10 g of Micromax Complete Trace Elements (Scotts Australia Pty Ltd, Baulkham Hills, NSW, Australia) and 10 g of Librel FE Chelated Iron (CIBA Specialty Chemicals Pty Ltd, Thomastown, VIC, Australia). All ingredients were thoroughly mixed and the pH adjusted to 6.5 with agricultural lime if required.

All plants were grown in controlled environment growth chambers (Phoenix Research, Edwardstown, SA, Australia) at the Plant Research Centre of the South Australian Research and Development Institute (SARDI). The rooms were lit by 10 400-W metal halide lights with a 14 h photoperiod and temperature was set at 18°C.

3.2.2 Scald Mutants

Strain 5 mutants: In total, 453 mutants of strain 5 were subjected to the screening program. These mutants were generated by the REMI mutagenesis method using plasmid pAN7-1 as the molecular tag and 20 units of restriction enzymes *Bam*HI, 363 mutants, or *Hind*III, 90 mutants, respectively.

UK7 mutants: 81 mutants of strain UK7 generated by Dr. Sylvie Albert at the Max-Plank-Institute for Plant Breeding Research, Cologne, Germany, were screened. These mutants also resulted from a REMI transformation with pAN7-1 as the molecular tag and 20 or 50 units of the restriction enzymes *Bam*HI and *Hind*III (Table 3.1).

Table 3.1: Restriction enzymes and concentrations used for the 81 UK7 mutants inoculated.

Restriction Enzyme	Enzyme Amount (units)	Mutants Inoculated
*Bam*HI	20	21
*Bam*HI	50	19
*Hind*III	20	25
*Hind*III	50	16

3.2.3 Inoculation Procedures

During the screening process, two inoculation methods were used - a rapid inoculation procedure utilising cotton balls and a more sensitive, but time consuming inoculation procedure that involved the production of a defined spore suspension. In each inoculation the wild type fungal strain and an uninoculated plant were included as positive and negative controls, respectively.

3.2.3.1 Cotton Ball Inoculations

Fungal material was inoculated onto the primary leaf of 10-d old barley seedlings, grown in an individual pot in potting mix, in the following way: a cotton ball was dipped into a 0.001% solution of Tween 20, rubbed over the surface of a 10-d old fungal culture, grown on Lima bean agar, and rubbed gently onto both sides of the barley leaves. Seedlings were then transferred to an opaque plastic box and misted with water. Pot density within the box was limited to avoid cross-contamination between seedlings. Furthermore, a high humidity environment was established within the box by covering its walls, floor and lid with water by spraying each surface with an atomiser. Once sealed, the inoculated plants were incubated for 16-24 hours in the dark. Following incubation, the lid of the box was partially opened to allow its internal environment to equilibrate with the external environment. Several hours later the pots were removed to benches in the controlled environment chamber.

3.2.3.2 Spore Inoculations

Pots containing 6-10 ten-day old barley seedlings were directly sprayed with 2 ml of a 0.001% Tween 20 solution containing approximately 5 x 10^6 spores/ml. Following inoculation, the plants were incubated in the same manner as described for cotton ball inoculations.

3.2.4 Non-pathogenic Mutant Identification

A 2-point scoring system was implemented to identify non-pathogenic mutants. Any mutants that produced symptoms consistent with the plant inoculated with the wild type fungal strain were discarded from the screening program. Conversely, any mutants that produced no symptoms were recorded and stored for further investigation (Fig 3.1).

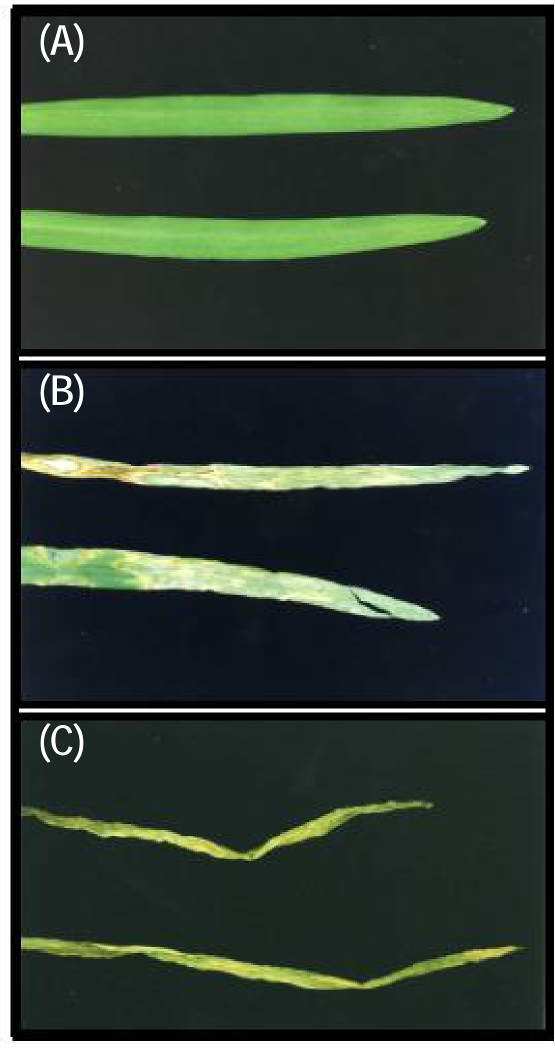


Figure 3.1: The 2-point scoring system used to identify non-pathogenic mutants. (A) Uninoculated control leaves and leaves 2 weeks post-inoculation with wild type strains 5 (B) and UK7 (C). Mutants that produced symptoms consistent with their wild type parental strains were considered pathogenic. Mutants that produced no symptoms and reflected the appearance of the uninoculated control were considered non-pathogenic.

3.2.5 Fungal Storage

To ensure long term viability, fungal mutants were stored cryogenically in liquid nitrogen. The procedure for storing fungal material was as previously reported (Lehnackers and Knogge, 1990) with the following alterations: agar plaques were frozen to –80°C at a rate of –1°C/min using a NALGENE –1°C Cryo Freezing Container (Nalge Nunc International, Rochester, NY, USA) and Diploma Instant Skim Milk Powder (Bonlac Foods Ltd, Mount Waverley, VIC, Australia) was used as a substitute for Difco Bacto Skim Milk in the cryoprotectant solution. Once preserved in this medium, virulence is considered stable and genetic uniformity is maintained.

3.3 Results

3.3.1 UK7 Mutants

To identify non-pathogenic mutants of strain UK7, a three-step screening process was implemented. The first and second rounds of screening utilised the rapid cotton ball inoculation technique. In the first round, 81 UK7 mutants were inoculated. Of these mutants, 24 mutants were identified as pathogenic and discarded as their disease reaction was sufficiently similar to the reaction observed on plants inoculated with the wild type strain UK7. The remaining 57 mutants did not produce disease symptoms and were subsequently subcultured. However, 14 of the 57 mutants would no longer grow in culture; this loss of viability was possibly due to successive rounds of subculturing leading to deterioration of the individual mutants. The 43 mutants that could be successfully subcultured were stored cryogenically. Furthermore, the initial rapid inoculation procedure was repeated with these mutants and a further 24 mutants showed full pathogenicity. Again these mutants were discarded from the screening program and the 19 remaining mutants subcultured. Although no mutants were eliminated due to loss of viability, a bacterial contamination had overcome two of the 19 mutants. The remaining 17 mutants were inoculated using the more sensitive spray inoculation protocol. For this purpose, a spore suspension with an adequate titre (5 x 10^6 spores/ml) was prepared for each of the 17 mutants and inoculated onto barley leaves. In total, 15 of the remaining mutants were deemed pathogenic due to the disease reaction on the inoculated plants. However, the remaining two mutants mimicked the uninoculated control plant showing no disease symptoms and were hence deemed non-pathogenic (Fig 3.2).

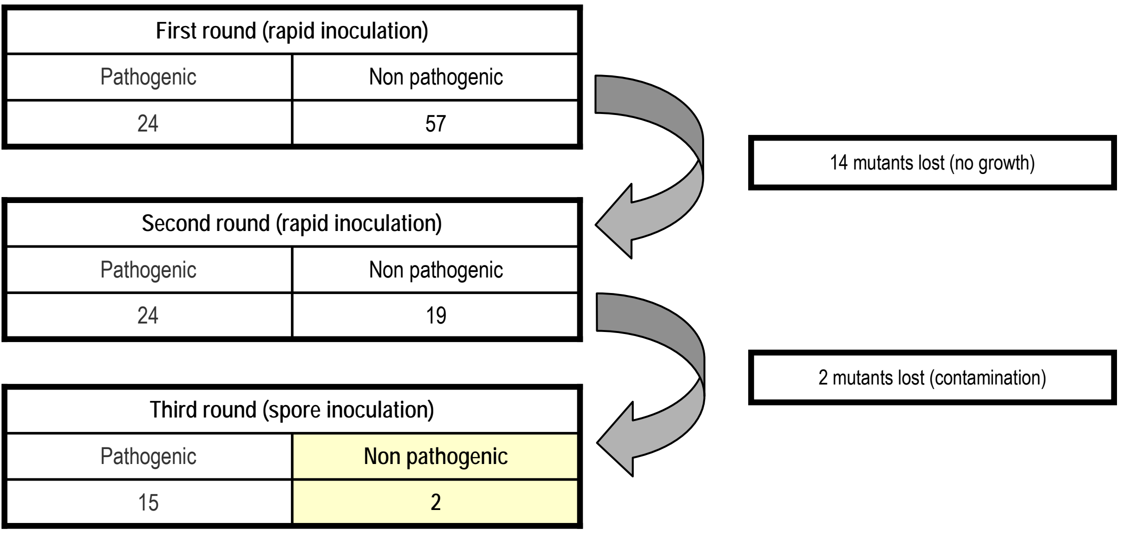
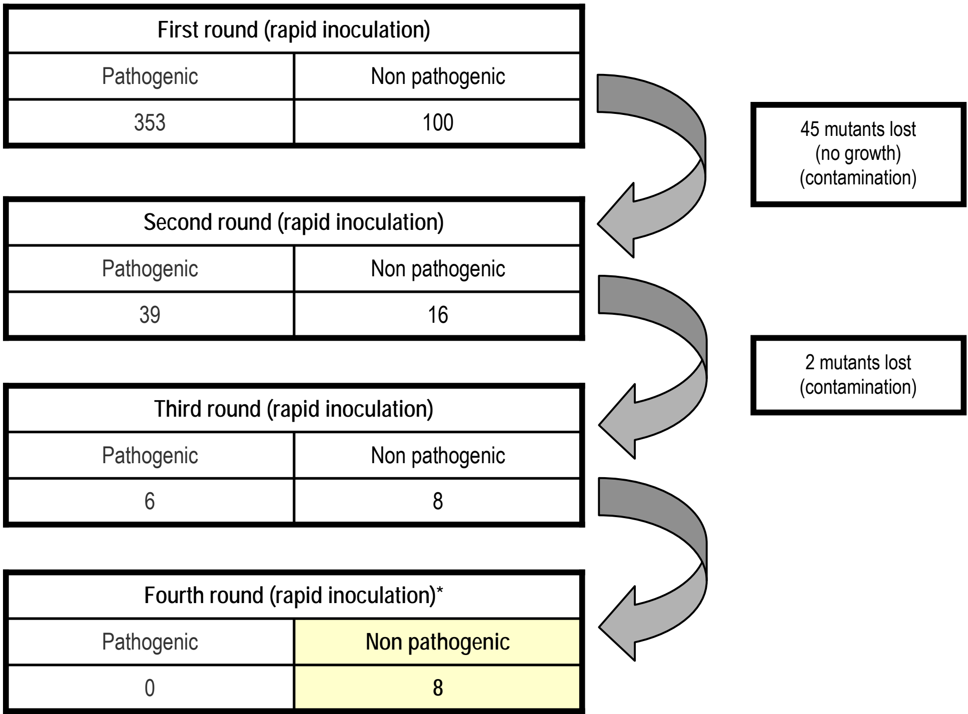


Figure 3.2: Summary of the screening process involving the 81 UK7 mutants.

4.3.2 Strain 5 Mutants

To identify non-pathogenic mutants of strain 5 the inoculation process described in section 3.2.3 was repeated using a total of 453 strain 5 mutants. The first rapid inoculation showed that 353 mutants caused disease symptoms sufficiently similar to the control plant inoculated with the wild type strain 5 to be classified pathogenic. After all pathogenic mutants were discarded, the remaining 100 mutants were subcultured. Unfortunately 45 of these mutants were either contaminated by a foreign agent or had become inviable due to a loss of fitness incurred by successive rounds of subculturing. However, the remaining 55 mutants were stored cryogenically and re-inoculated in a second rapid inoculation. Thirty-nine of the remaining mutants were identified as pathogenic due to their disease symptoms and discarded immediately. The 16 mutants that remained after two rounds of inoculations were again subcultured resulting in a further loss of two mutants due to contamination by a foreign microbe. However, the remaining 14 mutants failed to produce enough spores for a spray inoculation even though they were growing well as indicated by normal fungal biomass. Four cotton ball inoculations with low sporulating strain 5 produced symptoms equivalent to strain 5 spore inoculations (not shown) indicating that a lack of conidia production could not be correlated to a loss of fungal pathogenicity. Consequently, the rapid inoculation of any suspected non-pathogenic mutants was repeated a further four times. These rapid inoculations showed six mutants that produced pathogenic phenotypes and eight

mutants that did not produce disease symptoms when compared to the inoculated and uninoculated controls. All individual mutants produced the same symptoms in each repeat experiment. This result was considered as conclusive evidence that the eight mutants unable to successfully colonise the host were non-pathogenic (Fig 3.3).



*The fourth round (rapid inoculation) was repeated in quadruplicate, all experimental repetitions produced identical results.

Figure 3.3: Summary of the screening process involving the 453 strain 5 mutants.

3.4 Discussion

3.4.1 Frequency of Non-Pathogenic Mutants

In total 534 mutants were inoculated onto the universally susceptible barley host cultivar Sloop. From these mutants, 10 were identified as incapable of producing disease symptoms, two from the 81 UK7 mutants and eight from the 453 strain 5 mutants. These results indicate that non-pathogenic mutants were identified at a frequency of 2.47% and 1.77% in the fungal strains UK7 and 5, respectively. The percentage of non-pathogenic mutants identified in this study was

comparable with the previously identified 2-3% of non-pathogenic mutants (S. Albert, unpublished) for the strain UK7. Although strain 5 produced non-pathogenic mutants at a lower frequency than UK7 the frequency difference was negligible. The loss of mutants between inoculation steps in the screening process may have contributed to a decrease in non-pathogenic mutant frequency. In total 16 UK7 mutants and 47 strain 5 mutants were eliminated from the screening process because they did not grow. Furthermore, these mutants were lost after at least one or more rounds of inoculation. Therefore, it is likely that the number of mutants that were non-pathogenic within this group was proportionally larger than the original percentage of non-pathogenic mutants because a large number of pathogenic mutants had already been removed from the population. However, it should be noted that large variations in the percentage of non-pathogenic mutants were observed in other studies on other fungi (Sweigard *et al.*, 1998; Balhadere *et al.*, 1999; Kahmann and Basse, 1999).

3.4.2 Pathogenicity of Strain 5 Mutants

In total, 10 mutants were identified as non-pathogenic using the screening program detailed in this chapter. Unfortunately, of those 10 mutants only two, originating from the parental strain UK7, produced spores suitable for the preparation of a quantified spore suspension for plant inoculations. The remaining eight mutants failed to produce conidia and, therefore, could not be analysed using a defined spore suspension. The inability to inoculate a quantified number of spores onto a plant initially complicated the analysis of the pathogenicity status of the eight strain 5 mutants. The rapid inoculation technique was originally designed to allow the screening of large numbers of mutants with a minimum amount of labour. The disadvantage of this procedure is that it does not allow a qualitative and quantitative assessment of the fungal material applied to the plants. Consequently, the uncertainty remains as to whether lack of symptom induction by the eight strain 5 mutants is due to the presence of no or too low inoculum or indeed to non-pathogenicity of the mutants. However, the consistent occurrence of pathogenic symptoms in the non-sporulating strain 5 wild type control and the consistent lack of symptom development following inoculation with the strain 5 mutants, in multiple independent experiments conducted with both wild type and mutant strains, can be considered as conclusive evidence that these mutants were non-pathogenic.

The occurrence of low sporulating strain 5 also raises the question: how are the initial stages of infection progressing? It is likely that low sporulating strain 5 has a different mode of infection through mycelia than the germ tube produced by spores. This question is not addressed in this thesis. However, within the context of the project the method the fungus was using to infect the plant was not considered critical. The low sporulating wild type produced consistent symptoms and there were mutants of that wild type that were non-pathogenic leaving the projects main goal of identifying pathogenicity genes still possible.

The low sporulation of strain 5 raises two questions: what is responsible for the loss of sporulation and is the loss of sporulation reversible? The next chapter examines how the reduction of sporulation was overlooked during fungal culture and inoculations, even though it is an excursion from the overall aim of the thesis.

Chapter 4 – Excursion: Assessment of Fungal Sporulation

4.1 Introduction

The final sensitive round in the process of identifying non-pathogenic fungal mutants required the availability of a high-titre spore suspension for the inoculation of susceptible host plants. As explained in the preceding chapter, the mutants of *R. secalis* strain 5 had virtually lost the ability to produce spores. This observation prompted an examination of possible factors influencing the process of fungal sporulation. The intention of the present study however was not to investigate this developmental process in detail but rather to try and answer two questions: (i) is it possible to identify factors crucial for sporulation and (ii) can the sporulation process be manipulated to reverse the apparent loss of sporulation.

Few studies have examined the sporulation of *R. secalis*, and the vast majority of them discuss sporulation activity *in planta*. Schein and Kerelo (1956) tested the sporulation of *R. secalis* on 16 different solid media and observed that fungal cultures on Lima bean agar yielded the highest number of spores after a culture period of 16 d. Speakman (1993) described the production of large volumes of spores for plant breeding field trials. However, in this study *R. secalis* strains were used that were capable of producing large amounts of spores on agar media. Taken together, little information is available on the sporulation behaviour of *R. secalis in vitro*. Speakman (1993) indicates that sporulation rates differ for different strains of *R. secalis*. Differences in sporulation between the two major strains used in this study, strain 5 and strain UK7, are obvious because UK7 mutants that were identified as non-pathogenic had retained the capacity to sporulate. The question was, therefore, whether the sporulation behaviour of the two parent strains differs over successive generations.

To the author's knowledge, no study exists that involved the manipulation of a strain of *R. secalis* that failed to produce spores. Skromne *et al.* (1995) transferred cultures of *Aspergillus niger* from nutritionally complete medium to medium lacking either a carbon or a nitrogen source to

successfully induce sporulation. In contrast, Adams *et al.* (1998) noted that starvation stress was not essential for the development of conidia by *A. nidulans*. Shinohara *et al.* (2002) used glucose deprivation to show that the *ccg-9* gene was upregulated in *Neurospora crassa* during starvation stress. The *ccg-9* gene encodes a trehalose synthase involved in the morphogenesis of asexual conidiophores.

The standard medium used to culture *R. secalis* on agar is complex containing Lima bean extract. Hence, it cannot be divided into individual, quantifiable components. To examine starvation stress in *R. secalis* it was necessary to find a medium that could be manipulated with respect to the amount of nitrogen and carbon present. Liquid cultures of *R. secalis* are carried out in Fries medium No. 3 (Wevelsiep *et al.*, 1991) that contains NH_4 tartrate and NH_4NO_3 as major nitrogen sources (in addition to yeast extract) and that is supplemented with sucrose and yeast extract as a carbon source. Hence, it was decided to use this medium as the base for an agar media to initiate a study examining the effect of starvation stress on sporulation rates of *R. secalis*. If starvation stress could alter the sporulation of strain 5, this may suggest a way to stimulate sporulation.

4.2 Materials and Methods

4.2.1 Fungal Strains

The origin of the two strains UK7 and strain 5 has been previously explained (see 2.2.1). However, to examine sporulation behaviour, sporulating and non-sporulating samples of strain 5 were required. The loss of sporulation observed in strain 5 occurred during successive subculturing. Hence, the samples that had endured the largest number of successive subcultures (7 rounds of subculturing) represented the non-sporulating samples whereas samples with little successive subculturing (2 rounds of subculturing) were selected as sporulating samples of strain 5. To verify the ability, or conversely, inability of the fungal strains to sporulate, the fungal material was examined under the microscope.

4.2.2 Quantification of Fungal Spores

The previously described method to isolate spores of *R. secalis* (see 2.2.4.4) was labour intensive and involved long preparation times. Here, spores needed to be obtained from a large number of samples and, consequently, a rapid method to quantify spores was devised. Each spore quantification was based on a fungal culture grown on an agar dish of a diameter of 90 mm. Hence, the surface area of the fungal material is consistent for each plate. To quantify the sporulation rate of a fungal sample, 1.5 ml of nanopure water were applied to the surface of the plate which was then thoroughly scraped with the end of a 10 ml plastic pipette. One ml of the fungal suspension was transferred to a 1.5 ml Eppendorf tube, mixed thoroughly and a 15 µl aliquot transferred to a Neubauer counting chamber of 0.1 mm (Hausser Scientific, Horsham, PA, USA) to determine the spore titre of the suspension.

4.2.3 Comparative Sporulation Study: UK7 and Strain 5

To determine the differences between strain 5 and UK7 in sporulation behaviour over successive generations, sporulating samples of strain 5 and UK7 were applied to Lima bean agar plates. One generation is recorded as one round of subculturing. After 10-d fungal material was isolated to quantify spores and inoculate a new agar plate. Fungal material used to inoculate plates for new cultures was not quantified. Following 10-d incubation, new cultures had completely covered the new plate indicating no further fungal growth was possible and a maximum area for conidia production.

4.2.4 Starvation Media

The two media used, Lima bean agar and supplemented Fries medium No. 3, have been described elsewhere (see 2.2.2). However, supplemented Fries medium No.3 was modified to investigate starvation stress. Supplemented Fries medium No. 3 consists of the following components: yeast extract (0.1% w/v), NaCl (1.711 mM), KH_2PO_4 (7.35 mM), $MgSO_4$ (2.03 mM), $CaCl_2$ (0.884 mM), sucrose (21.91 mM), ammonium tartrate (21.14 mM) and NH_4NO_3 (12.49 mM). To create starvation media, this medium was modified to contain a series of 1:2 dilutions of either sucrose or NH_4 tartrate and NH_4NO_3. In total, five different concentrations of the carbon and nitrogen

components were included creating a total of 25 experimental treatments. The carbon and nitrogen concentrations can be observed in Table 4.1.

Table 4.1: Carbon and nitrogen concentrations present in the starvation media. These concentrations are calculated from the amounts of sucrose or NH_4 tartrate and NH_4NO_3, included in Fries medium No. 3. Absolute carbon and nitrogen concentrations cannot be calculated because yeast extract contains some carbon and nitrogen sources. Yeast extract, however, is complex and cannot be separated into its individual components.

	Dilution Factor				
Dilutions	1	0.5	0.25	0.125	-
Carbon [mM]	21.91	10.96	5.48	2.74	0
Nitrogen [mM]	33.63	16.82	8.41	4.2	0

4.3 Results

4.3.1 Sporulation of Strains 5 and UK7 Over Successive Generations

Transformation of *R. secalis* for mutant generation according to the polyethylene glycol/$CaCl_2$ protocol and the production of inoculum for phenotypic analysis require a total of six steps (Table 4.2). For protoplast preparation, the original fungal material needs to be grown in liquid culture. After transformation the protoplasts are transferred to selective agar media. The remainder of the process then involves successive subculturing to multiply sufficient fungal material for storage and the raising of an adequate spore suspension for inoculation. Therefore, it was necessary to monitor the sporulation behaviour of *R. secalis* over six subculturing steps.

Table 4.2: Fungal transformation and the number of generations required to produce sufficient fungal material for a spore inoculation.

Generation					
I	II	III	IV	V	VI
Liquid Culture	Selective Media	Mutant Colony Isolation	Spore Multiplication	Spore Multiplication	Inoculation

The sporulation behaviour of strain 5 was examined after successive subcultures over 6 generations on lima bean agar. The strain 5 sample used to examine the changes in sporulation that occurred in successive generations was selected from the original stock of strain 5 that had been used to produce REMI mutants. The number of spores counted after each successive generation is presented in Table 4.3

Table 4.3: The average titres of spores from fungal strains 5 and UK7 in successive generations calculated from four independent experiments (replicates). Standard deviations are included for generations IV, V and VI.

	Generation					
	I	II	III	IV	V	VI
Strain 5	$2.1x10^6$	$3.4x10^6$	$2.8x10^6$	$1.4x10^6 \pm 0.1$	$0.88x10^6 \pm 0.18$	$0.0025x10^6 \pm 0.005$
UK7	n.d.*	n.d.*	n.d.*	$9.06x10^6 \pm 1.55$	$11.92x10^6 \pm 1.81$	$9.76x10^6 \pm 0.73$

*n.d. - no data recorded

The data presented in Table 4.3 revealed a rapid decrease in spore titres of strain 5 after the third generation. From the fifth generation on, the number of spores produced can be regarded as insufficient for inoculation experiments. Interestingly, no obvious change in fungal morphology was observed macroscopically over the course of the six generations.

To test if there were differences between strains 5 and UK7 after the 3rd generation, the above experiment was repeated in quadruplicate using UK7 that had passed three generations (Table 4.3; Figure 4.1).

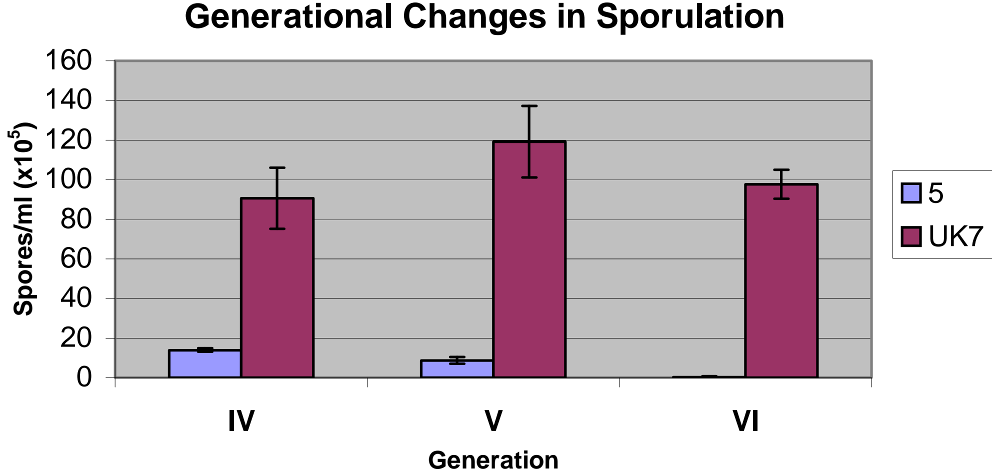


Figure 4.1: Sporulation of the two fungal strains, UK7 and 5 after the 4th, 5th and 6th generations. Bars indicate standard deviations of each column.

Two significant trends were apparent concerning the sporulation of strains 5 and UK7 become apparent (Fig 4.1). Firstly, the most obvious difference is that UK7 produces considerably more spores than strain 5 (approximately 10 times at the 4th generation). This difference is consistent for the first three generations (not shown). Secondly, no significant difference in sporulation of fungal strain UK7 was observed between generations 4, 5 and 6. In contrast, strain 5 shows a significant decrease in the number of spores produced over generations 4, 5 and 6. Consequently, it would be difficult to raise enough spores for the inoculation experiments from plates of strain 5 after the 4th generation.

4.3.2 Influence of N and C on Sporulation

Reports from other filamentous fungi suggest that conidiation can be encouraged by transferring hyphae from nutritionally complete media to media that lack a sufficient carbon or nitrogen source. A starvation study was therefore performed employing a non-sporulating sample of *R. secalis*

strain 5 and sporulating samples of strains 5 and UK7. In total 25 combinations of nitrogen and carbon concentrations were used in duplicate. Non-sporulating strain 5 was used in a preliminary starvation experiment to try and encourage sporulation of this strain. The initial amount of fungal material used in each experiment was not quantified for each fungal strain because an accurate quantification of the fungal material present in the non-sporulating strain 5 sample would be difficult. As a consequence, comparisons were not drawn between each table, or fungal sample, only the response of the individual samples was analysed. The spore counts made with each fungal sample, non-sporulating strain 5, sporulating strain 5 and strain UK7 are reported in Tables 4.4 - 4.6, respectively.

Table 4.4: Spore counts (spores/ml x10^5) registered after non-sporulating strain 5 was cultured on supplemented Fries medium No. 3 with the carbon (C) and nitrogen (N) concentrations adjusted. Note that 2 spore counts are given for each C/N ratio because the experiment was performed in duplicate.

	Nitrogen [mM]									
Carbon [mM]	33.63		16.82		8.41		4.2		0	
21.91	1	0.5	0.5	0	0	0.5	0.5	0	0	0
10.96	0	0	0	1	0	0	0	0	0	0
5.48	0	0	0	0	0.5	0.5	0	0	0.5	0
2.74	1	0	0	0	0.5	0	0	0	0.5	0
0	0	0	0	0.5	0.5	0	0	0	1	0.5

The adjusted C and N concentrations represented in Table 4.4 failed to have any significant effect on the sporulation rate of the non-sporulating *R. secalis* strain 5. None of the C/N ratios appear to cause a noticeable increase in sporulation. Two control plates with non-sporulating strain 5 grown on Lima bean agar gave spore counts of 0 and 0.5 x 10^5 spores/ml.

Table 4.5: Spore counts (spores/ml x 10^5) recorded after sporulating strain 5 was cultured on supplemented Fries medium No. 3 with the C and N concentrations adjusted. Two spore counts are recorded for each experimental level because the experiment was performed in duplicate.

	Nitrogen [mM]									
Carbon [mM]	33.63		16.82		8.41		4.2		0	
21.91	2.5	3	12.5	14	2.5	2	3	1	2.5	3
10.96	7	8	10	10	4.5	5.5	6.5	2.5	3	3
5.48	4	5	6.5	6	6	8	4.5	12	3.5	6
2.74	5	6	3.5	5	5	2.5	6.5	3.5	2.5	1.5
0	7	11	0.5	5.5	0.5	15	7.5	15	6.5	9

When a sporulating sample of strain 5 was used in the starvation experiment, there was again no correlation between C and N levels and sporulation. The spore counts observed for all C/N ratios were consistently lower than those of 2 control culture grown on Lima bean agar (19 x10^5 and 22x10^5 spores/ml).

Table 4.6: Spore counts (spores/ml x 10^5) recorded after strain UK7 was cultured on supplemented Fries medium No. 3 with the C and N concentrations adjusted. Note that 2 spore counts are given for each C/N ratio because the experiment was performed in duplicate.

	Nitrogen [mM]									
Carbon [mM]	33.63		16.82		8.41		4.2		0	
21.91	26.5	34.5	34	44.5	40	17	52.5	39.5	60	51.5
10.96	14	18	16	12.5	18.5	22.5	32	33.5	45	46
5.48	6	9	2	4	6	11	12	16.5	53	45.5
2.74	3	1.5	5	3	4.5	1.5	9	9.5	32	39
0	1	1	1	0.5	0.5	1	1.5	1.5	32	21

Control plates of strain UK7 cultured on Lima bean agar produced $89x10^5$ and 63.5×10^5 spores/ml. In comparison, the modified Fries medium No. 3 containing the full amount of C and N yielded approximately 40% of that number of spores (Table 4.6). Nevertheless, close examination of the table reveals two major trends. Firstly, the sporulation of strain UK7 appears to increase with decreasing amounts of N present in the medium. Secondly, there appears to be an overall decrease in the number of spores when the C concentration in the medium is lowered. These two trends were consistent over both UK7 experiments could not be observed with the sporulating strain 5 sample. Both trends are illustrated more clearly in Fig 4.3 where the spore counts from the duplicate experiments are averaged.

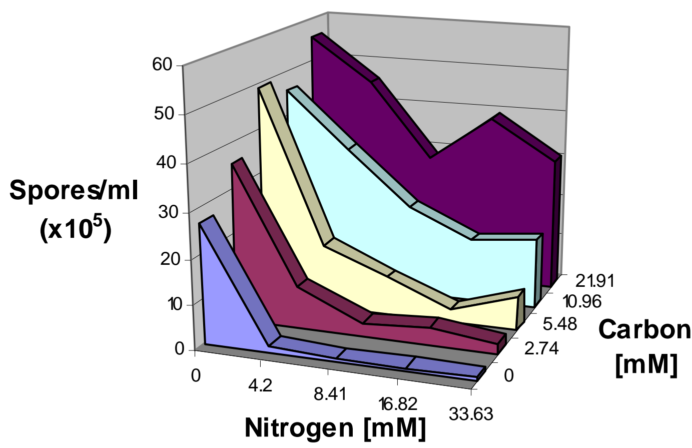


Figure 4.3: Sporulation *vs.* C and N concentration [mM]. Experimental data taken from Table 4.5 were averaged and presented graphically to show the influence of changing C and N concentrations on the sporulation of fungal strain UK7. Note that due to only duplicate experiments no statistical significance could be inferred from the data.

4.4 Discussion

Sporulation had been manipulated in other fungi by exposure to environmental stresses including starvation (Skromne *et al.,* 1995; Adams *et al.,* 1998; Shinohara *et al.,* 2002). Consequently, the effect of starvation stress on the sporulation of *R. secalis* was tested. However, manipulating fungal sporulation was complicated by the complex media required for fungal culture (Fries medium No.3 supplemented with yeast extract, containing both C and N, was the only medium available).

The starvation stress experiments were undertaken solely to determine whether the loss of fungal sporulation in strain 5 was reversible. C or N shortage did not affect conidia production in the sporulating sample of strain 5 nor did it revive sporulation in the non-sporulating sample of strain 5. In the response of strain UK7 to C/N starvation, two trends become apparent: sporulation increases with decreasing N concentrations and decreases with decreasing C concentration. This result is in agreement with Skromne *et al.* (1995), who showed that *A. nidulans* sporulates following transfer form nutritionally complete media to media deficient in a carbon or nitrogen source. Interestingly, even before the sporulation rate decreases, strain 5 generally produces substantially fewer spores than strain UK7, suggesting that conidiation is under different control in these strains. However, developmental regulation has not been investigated in *R. secalis* to date.

Different sporulation rates and metabolic changes during starvation stress imply that a group of genes is being differentially expressed. This immediately provides the opportunity to study the genetic control of sporulation in strain UK7. Given the importance of *R. secalis* as a plant pathogenic fungus, there is substantial interest in methods to control the spread of conidia and fungal proliferation on barley crops. Thus, elucidation of the genetic control of fungal proliferation may allow the identification or design of methods to control the spread of the fungus in the field.

The number of subculturing steps can severely interfere with sporulation, as was shown for strain 5. For this strain a statistically significant, dramatic decrease of spore formation occurred after 4 generations in culture. Following 4 generations, or subculturing steps, the fungus failed to produce a sufficient number of spores for inoculation experiments. In contrast, this trend is not manifested in strain UK7 that shows no difference in sporulation over six generations. When the generational change in sporulation of strain 5 is compared to the number of successive subculturing steps required for the phenotypic analysis of REMI mutants, it becomes obvious that

this strain is unsuitable for this type of research. At the inception of this project, such drastic loss of sporulation was not predicted nor anticipated and had not been observed with other *R. secalis* strains and, hence, was not considered a possibility. This study highlights the importance of monitoring both growth and sporulation in all experiments.

Chapter 5: Molecular Analysis of Non-Pathogenic Mutants

5.1 Introduction

Using the REMI approach 10 non-pathogenic *Rhynchosporium secalis* mutants were isolated. As a consequence of the insertional mutagenesis strategy the observed phenotypes are assumed to be directly linked to a molecular tag (Shuster and Bindel Connelley, 1999). The genomic sequences flanking the integration sites were isolated to identify putative genes that were affected by the integration event and to determine whether these genes have a function in fungal pathogenicity.

Several approaches were available to isolate flanking sequence. These approaches included thermal asymmetric interlaced (TAIL) PCR and plasmid rescue. TAIL-PCR has been successfully used to isolate flanking genomic sequences from fungal pathogens *Fusarium oxysporum* (Mullins *et al.*, 2001) and *Magnaporthe oryzae* (Li *et al.*, 2007) and plasmid rescue has been used to isolate genes from insertion mutants of the model fungus *Neurospora crassa* (Kothe and Free, 1998) and the phytopathogenic fungus *Cercospora nicotianae* (Chung *et al.*, 2003). While these methods have been successfully applied in other fungal organisms, PCR walking (Devic *et al.*, 1997) had been previously established and successfully used to isolate flanking sequence in *R. secalis* REMI mutants (S. Albert, unpublished).

Prior to identifying gene sequences disrupted by plasmid integration it is desirable to determine the number of integration sites present in the mutant. Mutants containing integrations at one site in the genome provide the most easily analysable material. *R. secalis* has no known sexual cycle and, therefore, plasmid integrations at multiple sites in the genome cannot be separated using crossing experiments (Goodwin *et al.*, 1994). As a consequence, mutants containing integrations at a single site in the genome represent the only material that can be reasonably analysed.

Following the identification of mutants with integrations at a single genomic location, integration structure must be analysed. Multiple copies of the molecular tag at a single genomic location complicate analysis because a PCR-based strategy with primers designed on the pAN7-1

plasmid are used to isolate flanking sequence. The presence of multiple copies of the plasmid increases the number of primer annealing sites reducing specificity of annealing and amplification at the desired plasmid-genomic DNA junction site. The number of integration sites in the genome and the structure of these integrations were analysed by Southern hybridisation.

PCR walking requires a primer site in the flanking unsequenced genomic DNA in combination with primers designed on the molecular tag, plasmid pAN7-1. To create a primer site in the flaking sequence a combination of restriction enzyme digests and ligations is employed (Devic *et al*., 1997). Firstly, blunt end restriction enzymes digest the mutant genomic DNA creating sites for adaptor ligation. The adaptor is a double stranded DNA molecule containing two annealing sites for the primer AP1 and the nested primer AP2 on the upper strand. The lower strand of the molecule is shortened by 36 bp such that it lacks the primer sequences and contains an amine group at its 3' end to prevent polymerase extension. Hence, no AP1 and AP2 primer binding sites are generated unless extension occurs from a primer annealed to the molecular tag or some other known sequence. Therefore, the exponential amplification of fragments containing the adaptor molecule ligated at each end is excluded.

This chapter outlines the isolation of approximately 2kb of genomic DNA flanking the integrated plasmid from the non-pathogenic mutants. This size was considered sufficient to identify the presence or absence of any putative gene within the region flanking the integration site. Furthermore, this DNA length was considered sufficient for performing loss-of-function studies by homologous recombination, should any gene be identified. The 10 *R. secalis* mutants' bioinformatic analysis will be presented in chapter 6.

5.2 Materials and Methods

5.2.1 Fungal Strains and Mutants

In total, 10 mutants and 2 wild type strains were used for analysis. The two wild type strains, 5 and UK7, have previously been described (Chapter 2). The mutants, their origins and nomenclature are listed below (Table 5.1).

Table 5.1: Origin of and nomenclature of the mutants subjected to molecular analysis.

Name	Wild Type Strain Origin	REMI Experiment		
		Enzyme	Concentration (units/transformation)	pAN7-1 Form
LH2013	UK7	*Hind*III	20	Linear
CH2034	UK7	*Hind*III	20	Circular
YH4.5	5	*Hind*III	20	Circular
YB4.20	5	*Bam*HI	20	Circular
YB4.44	5	*Bam*HI	20	Circular
YB5.3	5	*Bam*HI	20	Circular
YB5.9	5	*Bam*HI	20	Circular
YB7.319	5	*Bam*HI	20	Circular
YB7.395	5	*Bam*HI	20	Circular
YB7.412	5	*Bam*HI	20	Circular

5.2.2 Southern Analysis

The handling of materials and methods used in Southern hybridisations were described in chapter 2 (see 2.2.9 – 2.2.17). Only probe 1 was used for the work described in this chapter.

5.2.3 Polymerase Chain Reaction

5.2.3.1 Primer Design

PCR oligonucleotides were designed using the VectorNTi Version 7 software (Informax, Inc., Bethesda, MD, USA). Primer design was limited by the following parameters: GC content from 50 to 60 % and annealing temperature from 58 to 68°C. All primers were synthesised by Geneworks (Geneworks Pty Ltd, Hindmarsh, SA, Australia) using a 3900 DNA Synthesiser (Applied Biosystems, Scoresby, VIC, Australia). Each nested primer annealing site was positioned at least 50 bp from the end of the previous fragment to ensure that sequence overlap could be observed

between two walking fragments. The oligonucleotide primer sequences used during the course of this study are presented in the appendix.

5.2.3.2 PCR Amplification

PCR reactions were conducted using a Perkin Elmer GeneAmp® PCR System 9700 (Applied Biosystems, Scoresby, VIC, Australia) and the PCR products were separated on 1% agarose/TAE gels that were visualised under UV light following ethidium bromide staining.

Using different combinations of amplification programs and enzymes, PCR was used for the production of low and high fidelity PCR products of different lengths. In general, two types of products were desirable, short low fidelity fragments and longer high fidelity products.

5.2.3.3 Short Range Low Fidelity PCR

This form of PCR was used to amplify short (up to 3 kb) stretches of nucleotides. ABgene® Red Hot Polymerase (Integrated Sciences Pty Ltd, Willoughby, NSW, Australia) was used for these amplification reactions. The program used in this case was as follows:

95°C, 3 min.
94°C, 45 sec. ⎫
60°C*, 30 sec. ⎬ 30x
72°C, 1 min**. ⎭
72°C, 7 min.

*Step 3 represents the annealing temperature of the primers used in the reaction. This temperature was adjusted according to the primer pair used in the reaction.

**Step 4 represents the extension phase of the program *i.e.* the time taken for the polymerase to add nucleotides to the 3' end of the oligonucleotide primer. If a larger fragment of DNA was to be amplified, the extension time was increased accordingly.

5.2.3.4 High Fidelity PCR

To reduce the number of base pair substitutions in an amplification product a high fidelity or proofreading polymerase was employed. If short fragments were required then the Xpand high Fidelity Polymerase enzyme was used (Roche Diagnostics Australia Pty Ltd, Castle Hill, NSW, Australia) according to the manufacturer's instructions. The previously mentioned PCR program (see 5.2.3.3) was used in this case. However, if larger fragments of DNA were required (>3kb) the Gene Amp® XL PCR Kit (Applied Biosystems, Scoresby, VIC, Australia) was employed and reaction mixtures were set up according to the guidelines presented by the manufacturer. The long range PCR program amplified fragments of up to 5.5 kb and was as follows:

95°C, 5 min.
94°C, 30 seconds. } 30x
60°C, 6 min. }
72°C, 10 min.

Reaction products were separated on 1% agarose/TAE gels and any fragments of interest excised from the gel.

5.2.4 Genomic Walking

The protocol used in this project was adapted from the protocol established by Devic *et al.* (1997).

5.2.4.1 Genomic DNA Preparation-Adaptor Ligation

Two and a half micrograms of fungal genomic DNA were digested with 80 units of one of the following blunt end restriction enzymes: *Eco*RV, *Pvu*II, *Dra*I, *Sca*I, *Swa*I, *Sma*I or *Ssp*I (New England Biolabs, Ipswich, MA, USA) as per manufacturer's instructions. The cleaved DNA was purified by phenol/chloroform extraction and precipitated with 200 µl of 100% ethanol, 10 µl of Na acetate (3 M, pH 5) and 20 µg of glycogen (Invitrogen Australia Pty Ltd, Mount Waverley, VIC, Australia) and centrifuged at 13000 rpm for 10 min. The digested genomic DNA was then

resuspended in 20 µl of nanopure water. An adaptor duplex was then created by mixing adaptor oligonucleotide 1 (5' CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGGAGGT 3') with adaptor oligonucleotide 2 (5' ACCTCCCC 3') to a final equimolar concentration of 50 µM, heating in a boiling water bath for 1 min, and cooling slowly to room temperature. The adaptor duplex was added to 10 µl of the digested genomic DNA to a final concentration of 5 µM. Ten units of T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) were then added and the adaptor duplex and genomic DNA ligated according to the manufacturer's instructions in a final volume of 20 µl. The ligase was heat inactivated at 70°C for 10 min and the mix diluted ten-fold to give the final genomic DNA walking stock.

5.2.4.2 Primers and PCR protocols

Two nested primers, AP1 (5' GGATCCTAATACGACTCACTATAGGGC 3') and AP 2 (5' CTATAGGGCTCGAGCGGC 3') were used in all genomic walking reactions. Many different nested gene-specific primer pairs were used in the course of this study and are described in the appendix.

Two PCR reactions were required for each individual walk. The first PCR reaction (final reaction volume of 20 µl) contained the following final concentrations: primers (AP1 and the first of the nested gene specific primer pair) – 200 nM, deoxy-nucleotides (adenine, guanine, cytosine, thymine) – 200 µM (each), 2.5 ng DNA and 0.25 units of expand high fidelity polymerase (Roche Diagnostics Australia Pty Ltd, Castle Hill, NSW, Australia) with 1x expand high fidelity PCR buffer. The first PCR program was as follows:

94°C, 45 sec.
72°C, 4 min. } 7x
94°C, 45 sec.
67°C, 4 min. } 32x

The second PCR was essentially a repeat of the first with two modifications: the nested primers (AP2 and the nested gene specific primer) were used with 0.4µl of a 1:100 dilution of the product of the first PCR reaction as a template. The second PCR program was as follows:

94°C, 45 sec.
72°C, 4 min. } 5x
94°C, 45 sec.
67°C, 4 min. } 30x

Finally the products of both reactions were separated on a 1% agarose/TAE gel, stained with ethidium bromide, visualised under UV light and any fragments of interest, larger than 300 bp excised with a sterile scalpel blade from the gel.

5.2.5 Cloning of PCR Products

The products from high fidelity PCR and genomic walking reactions were often needed for sequencing or cloning. Once the desired amplification product had been excised from the gel using a clean scalpel blade under UV light, the DNA was purified using the QIAquick® Gel Extraction Kit (Qiagen Pty Ltd, Doncaster, VIC, Australia). Following purification, PCR products were ligated into the pGEM®-T Easy Vector System II (Promega, Sydney, NSW, Australia), following manufacturer's instructions. Ligated plasmids were then transferred to competent *E. coli* cells, strain DH5α Cells (see 2.2.5.2 – 2.2.5.3) and plated onto selective LB media.

To determine whether a cloning reaction had been successful, it was necessary to show that the plasmid containing the desired insert was present within a bacterial colony. Bacterial colonies growing on ampicillin LB agar were screened by short range, low fidelity PCR using the universal primer pair T7 (5-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAG-3'). The amplification product was separated on a 1% agarose/TAE gel, stained with ethidium bromide and visualised under UV light. A comparison of product size with the original PCR walk fragment was made and if the cloning reaction was considered successful, a liquid culture, LB broth and the antibiotic ampicillin (100 µg/ml), of the corresponding bacterial colony was grown for 16 h at 300 rpm and 37°C. Following incubation, a small-scale plasmid DNA extraction and purification was performed on the liquid culture using the Qiaprep® Spin Miniprep Kit (Qiagen Pty Ltd, Doncaster, VIC, Australia), following manufacturer's instructions.

Furthermore, liquid cultures of these colonies were converted to glycerol stocks (15% (w/v) glycerol) and stored at -80°C (Sambrook, *et al.*, 1989).

5.2.6 DNA Sequencing

DNA sequencing reactions were based on the BigDye® Terminator v3.1 (Applied Biosystems, Scoresby, VIC, Australia) sequencing mix and set up according to manufacturer's instructions. Sequencing data were generated by the Institute of Medical and Veterinary Sciences (Adelaide, SA, Australia) using a 3900 DNA Synthesiser (Applied Biosystems, Scoresby, VIC, Australia).

5.2.7 DNA Sequence Analysis

Individual genomic sequence fragments obtained were assembled into one sequence using the ContigExpress program of the Vector NTI Suite7.0 software (InforMax, Inc., Bethesda, MD, USA).

5.3 Results

5.3.1 Southern Analysis

To deduce the number and type of integrations present in each of the 10 non-pathogenic mutants, Southern analysis was carried out with the enzymes *Eco*RV and *Nco*I in combination with probe 1 (see 2.3.1.3). *Eco*RV cleavage, which does not occur within the pAN7-1 sequence, yields the number of separate integrations into the fungal genome. In contrast, *Nco*I, which cleaves at a single site in pAN7-1 within the region hybridising to probe 1 should provide information on multiple integrations in tandem array by yielding fragments of defined sizes (Fig 5.3). Non-pathogenic mutants were generated using two enzymes, *Hind*III and *Bam*HI, and therefore, the expected fragment pattern after *Nco*I digestion is dependant on the enzyme used to generate the REMI mutants. The location of probe 1 with respect to the sequence of plasmid pAN7-1 linearised with either *Bam*HI or *Hind*III can be observed in Fig 5.1.

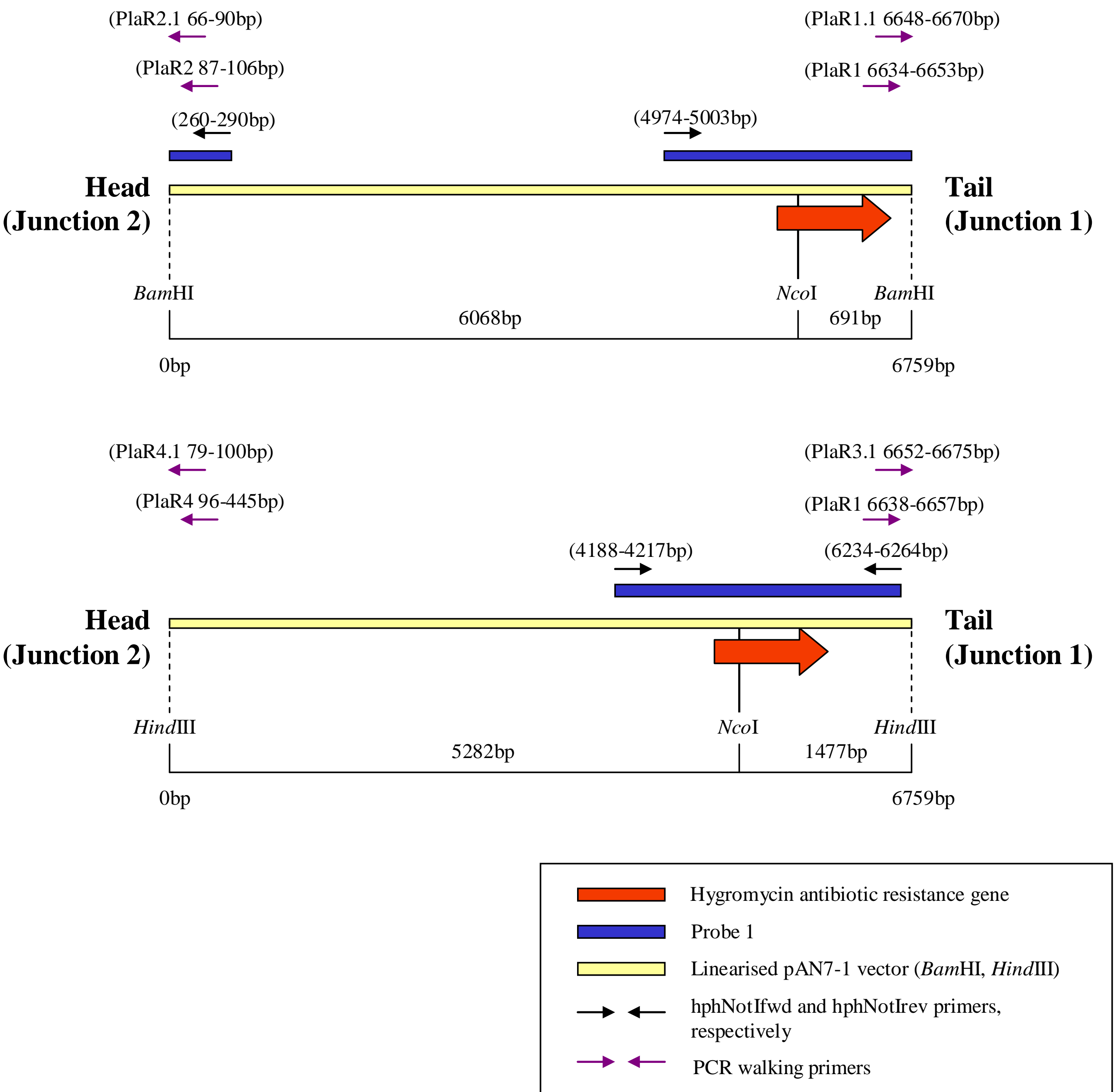


Figure 5.1: Location of probe 1, restriction sites and PCR walking primers used in the analysis of non-pathogenic REMI mutants. The diagram shows pAN7-1 after linearization with the restriction enzymes *Bam*HI (top) and *Hind*III (bottom).

In a *Bam*HI REMI mutant, one copy of pAN7-1 integrated at a single location within the fungal genome will produce two fragments larger than 6068 bp and 691 bp, respectively, upon digestion with *Nco*I and hybridisation with probe 1. In comparison, REMI mutants generated with

*Hind*III will produce two fragments larger than 5282 bp and 1477 bp. The autoradiographs of Southern hybridisation upon cleavage of DNA from the 10 REMI mutants using *Eco*RV and *Nco*I, respectively, are presented in Fig 5.2.

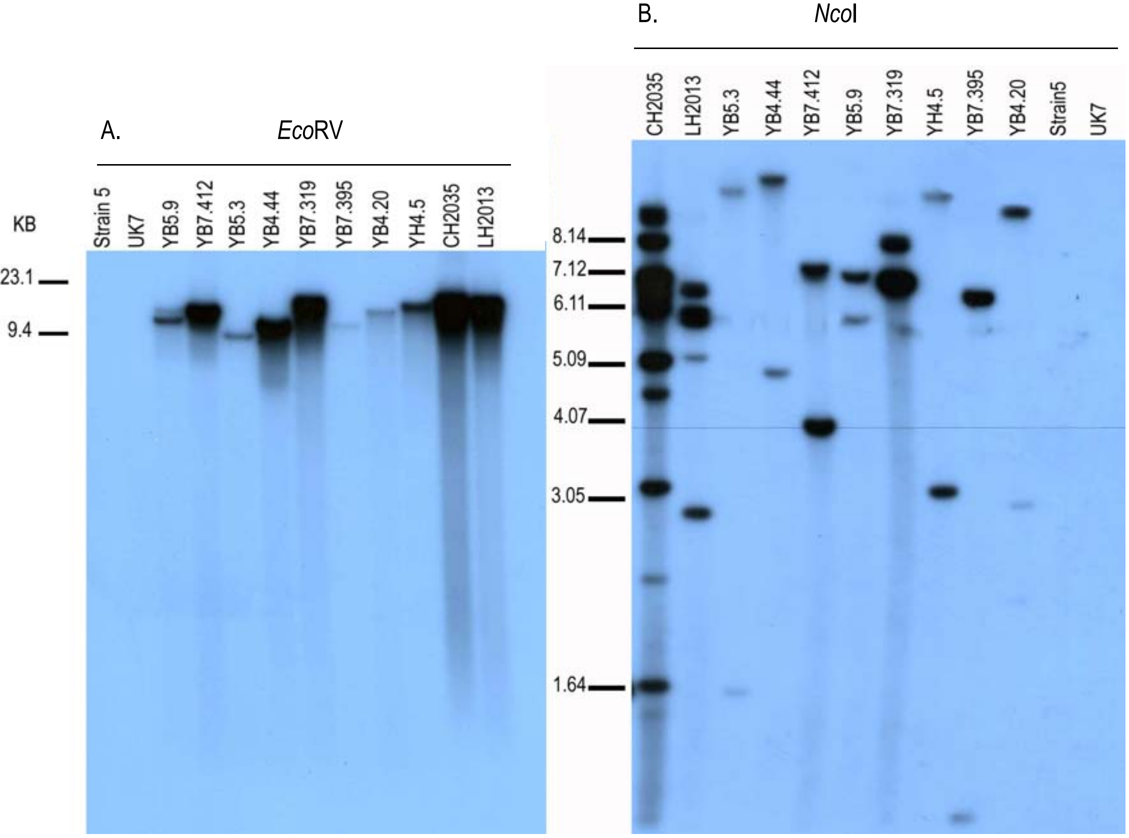
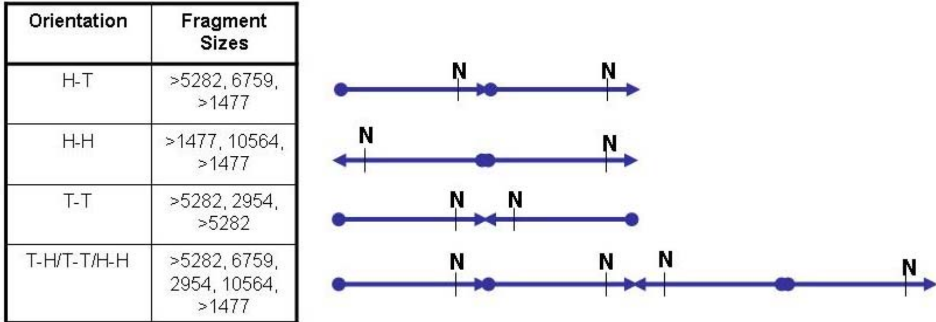


Figure 5.2: Southern analysis of DNA from the 10 non-pathogenic REMI mutants cleaved with the restriction enzymes *Eco*RV and *Nco*I and hybridised with probe 1.

Mutants YB5.3, YB4.44, YB7.412, YB5.9, YB7.319, YH4.5, YB4.20 and YB7.395 give fragment sizes consistent with a pAN7-1 integration at a single genomic location when cleaved with the restriction enzymes *Eco*RV and *Nco*I. Interestingly these eight mutants all originate from fungal strain 5 whereas the two remaining mutants originating from the UK7 strain show more complex banding patterns. UK7 mutants CH2034 and LH2013 show a single band upon digestion with *Eco*RV indicating that integration has occurred at a single location within the fungal genome. However, upon *Nco*I digestion DNA from these mutants produced 12 and 4 separate fragments, respectively. If multiple copies of the vector had integrated at a single genomic location in tandem

array then, a maximum of five different bands should be visible depending on the number and orientation of the integrated plasmid copies (Fig 5.3).



Orientation	Fragment Sizes
H-T	>5282, 6759, >1477
H-H	>1477, 10564, >1477
T-T	>5282, 2954, >5282
T-H/T-T/H-H	>5282, 6759, 2954, 10564, >1477

Figure 5.3: Possible orientations upon tandem integrations of two or more plasmid copies at a single genomic site and the number and length of fragments obtained upon *Nco*I cleavage. Arrows represent pAN7-1 molecules linearised with *Hind*III. Abbreviations: H = head; T = tail; N = *Nco*I restriction enzyme site.

Five fragments are released upon *Nco*I digestion if four linearised pAN7-1 molecules integrate in the orientation presented in Figure 5.3. These molecules would then be identified by hybridisation with probe 1: three fragments of 2954 bp, 10564 bp and 6759 bp and two fragments longer than 5282 bp or 1477 bp. Although extensive Southern analysis may have helped to explain the complex banding patterns obtained with the mutants CH2034 and LH2013, technical difficulties associated with Southern hybridisations excluded this possibility due to time constraints.

All mutants contained integrations at only one genomic location and therefore were suitable for further analysis. However, two problems prevented the analysis of every individual non-pathogenic mutant. Firstly, multiple plasmid integrations in tandem array reduce PCR walking efficiency and, secondly, the labour required to analyse all of the non-pathogenic mutants would exceed the time frame of this dissertation. Consequently, further analysis was restricted to the six mutants: YB4.20, YB4.44, YB7.395, YB7.412, YH4.5 and LH2013. Two of the *Hind*III mutants were chosen, YH4.5 and LH2013, and four *Bam*HI generated REMI mutants, YB4.20, YB4.44, YB7.395 and YB7.412, were selected at random from *Bam*HI mutants showing simple single integrations. Mutant YH4.5 was selected because it showed a simple single integration but also

because it was generated with *Hind*III during transformation. Mutant LH2013 was selected even though Southern analysis indicated a complex integration site because it originated from a different wild type strain, UK7, than the other mutants.

5.3.2 PCR Walking

PCR walking using DNA from the six selected non-pathogenic mutants identified the sequences flanking the integration sites. Each walk fragment was sequenced and compared to previous fragments to show sequence overlap. Furthermore, fragment overlap was verified using separate PCR check fragments that spanned the region where walking fragments overlapped. PCR walking occurred in a stepwise fashion for each mutant and therefore each mutant will be discussed individually in this section. The regions of 2-fold sequence redundancy will be indicated for each mutant. All primers used in mutant analysis and their respective locations are presented in the appendices. All DNA sequences are presented in the appendices.

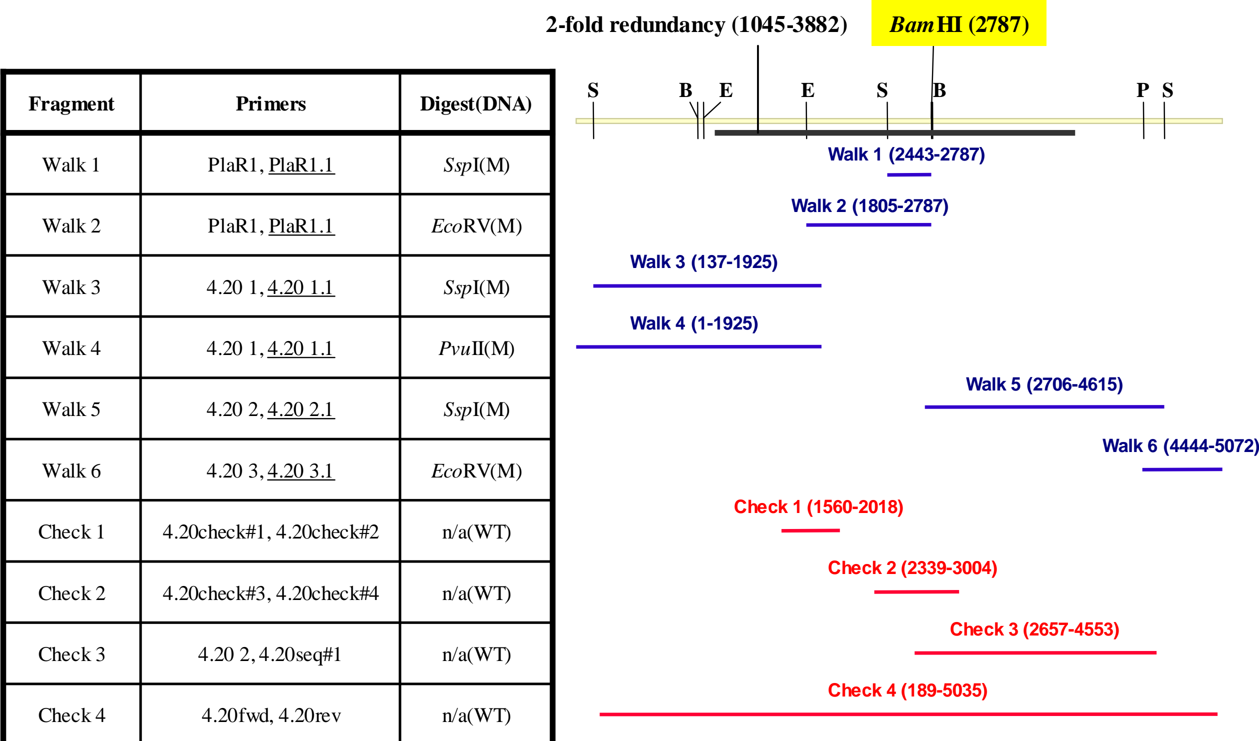
5.3.2.1 Mutant YB4.20

Figure 5.4 shows the location and length of fragments amplified by either general PCR (Check fragments) or PCR walking (Walk fragments) in the approach to elucidate the sequence surrounding the integration site in the DNA of mutant YB4.20.

Initially, primers PlaR1 and PlaR1.1 were used to walk from the pAN7-1 integration site into the fungal genome. Two separate walking reactions yielded fragments walk-1 and -2 that amplified from the integration to the *Ssp*I (2443) and *Eco*RV (1805) restriction sites, respectively. The walk-2 fragment was used to design primers 4.20 1 and 4.20 1.1 that amplified two further fragments, walk-3 and walk-4, in two separate reactions. Walk-3 amplified a fragment from the 4.20 1.1 primer to an *Ssp*I (137) restriction site. The walk-4 fragment was amplified from a wild type strain 5 genomic DNA digest, which had been treated with the restriction enzyme *Pvu*II.

The PCR walking primers PlaR2 and PlaR2.1 failed to amplify a fragment on the opposite flank of the pAN7-1 integration. Consequently, the walk-2 fragment was used to design the PCR walking primers 4.20 2 and 4.20 2.1 such that extension was oriented in the direction of the *Bam*HI restriction site, into which the pAN7-1 plasmid had integrated. In this case, strain 5 wild type DNA

was used as template for walking and the walk-5 fragment generated from the 4.20 2.1 primer site to an *Ssp*I (4615) restriction site. Finally, the walk-5 fragment was used to design the walking primers 4.20 3 and 4.20 3.1 that generated the walk-6 fragment from primer 4.20 3.1 to an *Eco*RV site.



Fragment	Primers	Digest(DNA)
Walk 1	PlaR1, PlaR1.1	*Ssp*I(M)
Walk 2	PlaR1, PlaR1.1	*Eco*RV(M)
Walk 3	4.20 1, 4.20 1.1	*Ssp*I(M)
Walk 4	4.20 1, 4.20 1.1	*Pvu*II(M)
Walk 5	4.20 2, 4.20 2.1	*Ssp*I(M)
Walk 6	4.20 3, 4.20 3.1	*Eco*RV(M)
Check 1	4.20check#1, 4.20check#2	n/a(WT)
Check 2	4.20check#3, 4.20check#4	n/a(WT)
Check 3	4.20 2, 4.20seq#1	n/a(WT)
Check 4	4.20fwd, 4.20rev	n/a(WT)

Figure 5.4: The location of walk and check fragments and the enzymes and primers used to generate those fragments in the genomic map of mutant YB4.20. The *Bam*HI restriction enzyme site highlighted indicates the pAN7-1 integration site. DNA abbreviations: (M) – mutant DNA; (WT) wild type or parental DNA; (n/a) – no restriction enzyme used. Restriction enzyme site abbreviations: B – *Bam*HI; E – *Eco*RV; P – *Pvu*II; S – *Ssp*I. Restriction enzyme sites *Pvu*II and *Eco*RV restriction sites present at the ends of fragments walk-4 and walk-6, respectively, are not marked in Fig 5.4.

In total, four 'check' fragments were amplified and sequenced to confirm walk fragments overlap. The check-1 fragment, amplified with primers 4.20check#1 and 4.20 check#2, verified the overlap of the walk-2, -3 and -4 fragments. The check-2 fragment was amplified with primers

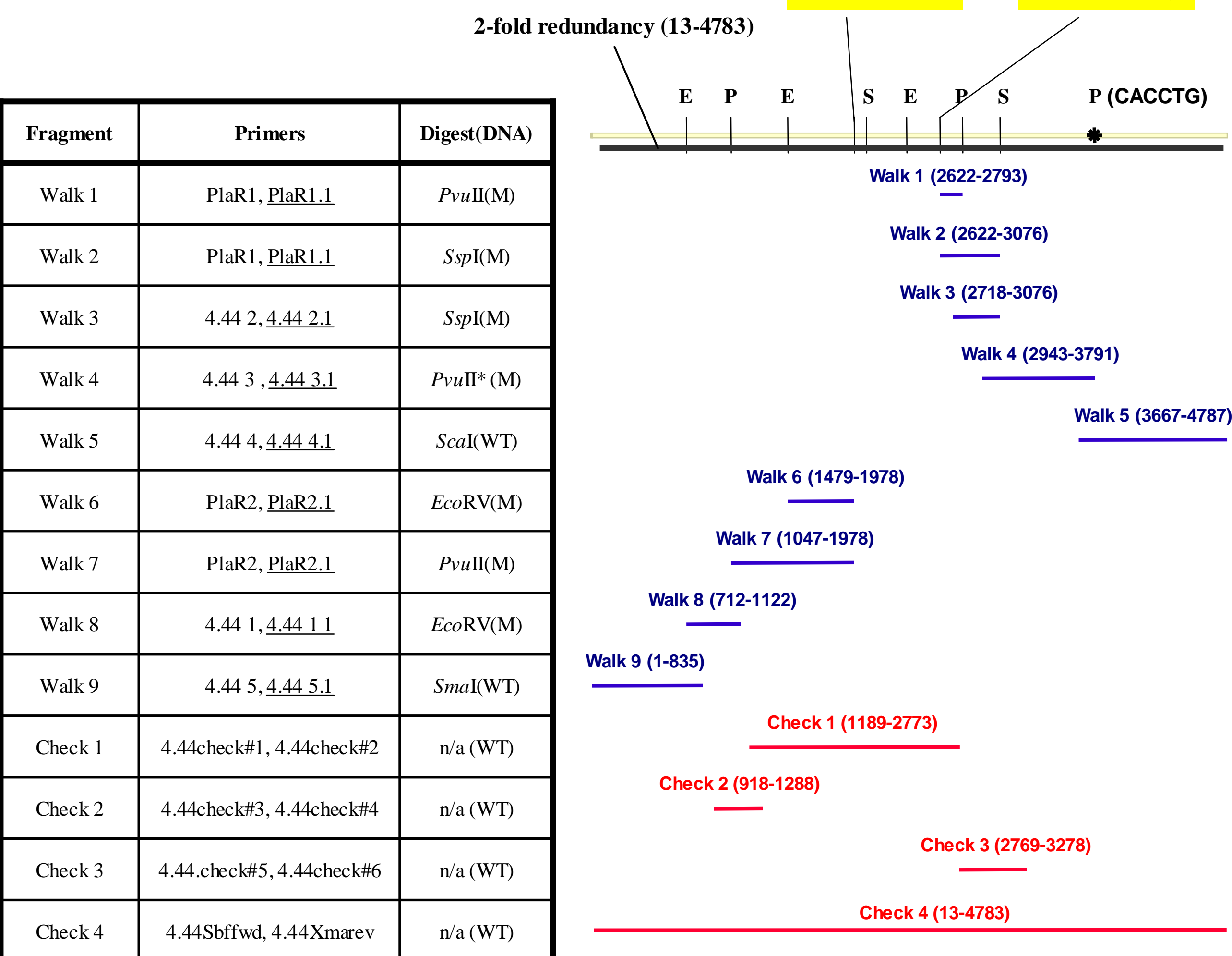
4.20check#3 and 4.20check#4 and verified the overlap between fragments walk-2 and -5. The check-3 fragment, amplified with primers 4.20 2 and 4.20seq#1, further confirmed this result overlapping the walk-2, -5 and -6 fragments. Finally, the check-4 fragment was amplified with primers 4.20fwd and 4.20rev that spanned the entire deduced sequence flanking the integration site of mutant YB4.20.

5.3.2.2 Mutant YB4.44

All PCR fragments used to elucidate the sequence surrounding the integration site in the genome of mutant YB4.44 are shown below (Fig 5.5). Integration of the pAN7-1 plasmid replaced the genomic fragment between two *Bam*HI sites located at 1978 bp and 2622 bp, respectively.

The walking primers PlaR1 and PlaR1.1 produced walk fragments 1 and 2 to the restriction sites *Pvu*II (2793) and *Ssp*I (3076), respectively. Initially, the walk-1 fragment was used to design the 4.44 2 and 4.44 2.1 primer pair that amplified the walk-3 fragment. However, the walk-3 fragment only amplified to the *Ssp*I (3076) restriction site previously reached by the walk-2 fragment. Consequently, new primers, 4.44 3 and 4.44 3.1, were designed and used to amplify the walk-4 fragment from *Pvu*II-digested mutant DNA. Interestingly, the *Pvu*II restriction site palindrome (CAGCTG) was not found where the walk-4 fragment terminated. Instead, the hexanucleotide sequence CACCTG was observed at the expected *Pvu*II location (Fig 5.5). The walk-4 fragment was used to design the primers 4.44 4 and 4.44 4.1 that amplified the walk-5 fragment to a *Sca*I restriction site.

The PlaR2 and PlaR2.1 walking primer pair are located within the pAN7-1 vector sequence (Fig 5.1) and were used to produce two walking fragments, -6 and -7, that terminated at *Eco*RV (1479) and *Pvu*II (1047) restriction sites, respectively. The latter of the two fragments, walk-7, was used to design the primers 4.44 1 and 4.44 1.1. The nested primer 4.44 1.1 amplified the walk-8 fragment to the *Eco*RV (712) restriction site and was used to design the nested primer pair 4.44 5 and 4.44 5.1 that amplified the walk-9 fragment.



Fragment	Primers	Digest(DNA)
Walk 1	PlaR1, PlaR1.1	*Pvu*II(M)
Walk 2	PlaR1, PlaR1.1	*Ssp*I(M)
Walk 3	4.44 2, 4.44 2.1	*Ssp*I(M)
Walk 4	4.44 3 , 4.44 3.1	*Pvu*II* (M)
Walk 5	4.44 4, 4.44 4.1	*Sca*I(WT)
Walk 6	PlaR2, PlaR2.1	*Eco*RV(M)
Walk 7	PlaR2, PlaR2.1	*Pvu*II(M)
Walk 8	4.44 1, 4.44 1 1	*Eco*RV(M)
Walk 9	4.44 5, 4.44 5.1	*Sma*I(WT)
Check 1	4.44check#1, 4.44check#2	n/a (WT)
Check 2	4.44check#3, 4.44check#4	n/a (WT)
Check 3	4.44.check#5, 4.44check#6	n/a (WT)
Check 4	4.44Sbffwd, 4.44Xmarev	n/a (WT)

Figure 5.5: Location of walk and check fragments and the enzymes and primers used to generate those fragments in the genomic map of mutant YB4.44. pAN7-1 was integrated between the highlighted *Bam*HI restriction enzyme sites. The genomic DNA between the highlighted *Bam*HI sites was deleted as a consequence of the integration process. DNA abbreviations: (M) – mutant DNA; (WT) wild type or parental DNA; (n/a) – no restriction enzyme used. Restriction site abbreviations: E – *Eco*RV; P – *Pvu*II; S – *Ssp*I. The *Pvu*II site marked with an asterisk deviated from the normal *Pvu*II restriction palindrome by a single base pair (underlined) suggesting star activity (see 5.3.3). *Sca*I and *Sma*I restriction sites present at the respective terminal fragment ends of walk-5 and walk-9 are not marked in Fig 5.4.

To verify walk fragment overlap, the following PCR fragments were generated and sequenced. The check-1 fragment amplified with primers 4.44check#1 and 4.44check#2 overlapped walk-1, -2 and -3, and walk-6 and -7. Furthermore, the check-1 fragment revealed that a 644 bp fragment was deleted from the genomic sequence where pAN7-1 integrated. The check-2 fragment, amplified with primers 4.44check#3 and 4.44check#4, independently verified the overlap between walking fragments walk-7 and -8 and the check 3 fragment, amplified with primers 4.44check#5 and 4.44check#6, proved the overlap of walk fragments walk-2, -3 and -4. Finally, the check-4 fragment was amplified with primer pair YB4.44Sbffwd and YB4.44Xmarev and sequenced reconfirming all overlapping fragments.

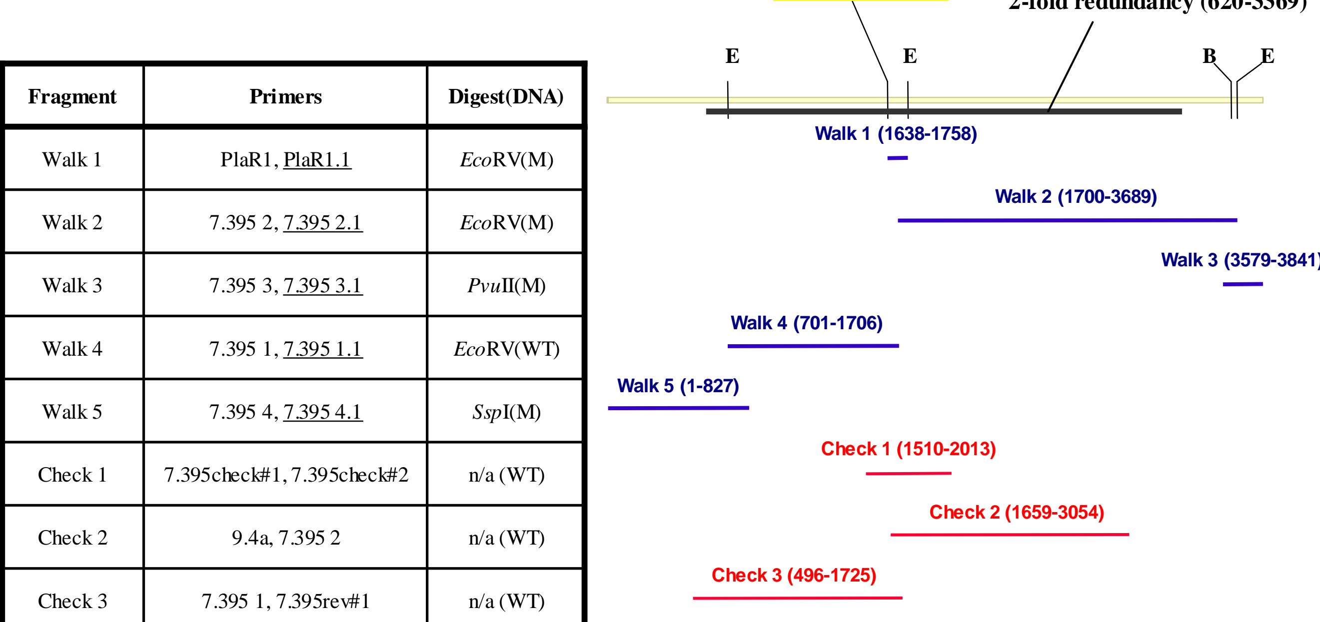
5.3.2.3 Mutant YB7.395

The location and length of fragments amplified and used to deduce the sequence flanking the integration site in the genome of mutant YB7.395 is shown below (Fig 5.6). The first walk fragment, walk-1, was amplified with the PlaR1 and PlaR1.1 primer pair and spanned the distance between the PlaR1.1 primer and the *Eco*RV restriction site (1758 bp). The walk-1 fragment was used to design the nested primer pair 7.395 2 and 7.395 2.1 that amplified the walk-2 fragment from primer 7.395 2.1 to the *Eco*RV restriction site (3689 bp). The walk-2 fragment was then used to design the primer pair 7.395 3 and 7.395 3.1 that successfully amplified the walk-3 fragment that eventually terminated at a *Pvu*II site.

The primers on the opposite flank of the integration, PlaR2 and PlaR2.1, failed to produce a fragment. Hence, new primers were designed from the walk-1 fragment and oriented such that extension occurred in the direction of the integration. However, in this case the wild type strain 5 DNA was used as template for walking. The nested primer pair 7.395 1 and 7.395 1.1 amplified the walk-4 fragment from the nested of the two primers, 7.395 1.1, to the *Eco*RV site located at 701 bp. The walk-4 fragment was then used to design the walking primers 7.395 4 and 7.395 4.1 that produced a walking fragment approximately 2.5kb in length (data not shown). Although initial sequencing produced only 827 bp (shown in Fig 5.6 as walk-5), it was decided that sufficient sequence was available for analysis.

In total, three check fragments were amplified. The check-1 fragment, amplified with primers 7.395check#1 and 7.395check#2, proved the overlap between the walk-1, -2 and -4

fragments. The check-2 fragment, amplified with primers 9.4a and 7.395 2, confirmed this overlap further and the check-3 fragment, amplified with 7.395 1 and 7.395 rev#1, confirmed the overlap between the walk-4 and -5 fragments.

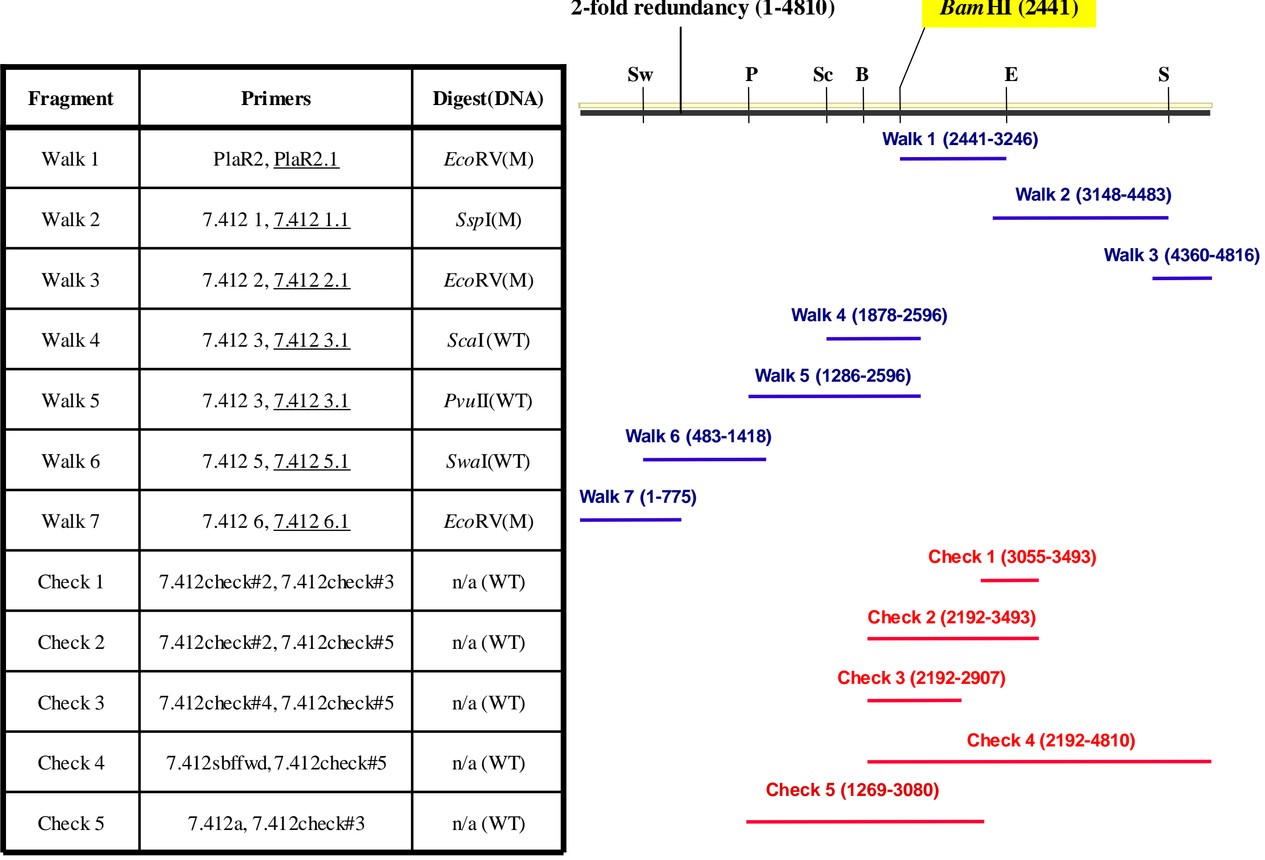


Fragment	Primers	Digest(DNA)
Walk 1	PlaR1, PlaR1.1	*Eco*RV(M)
Walk 2	7.395 2, 7.395 2.1	*Eco*RV(M)
Walk 3	7.395 3, 7.395 3.1	*Pvu*II(M)
Walk 4	7.395 1, 7.395 1.1	*Eco*RV(WT)
Walk 5	7.395 4, 7.395 4.1	*Ssp*I(M)
Check 1	7.395check#1, 7.395check#2	n/a (WT)
Check 2	9.4a, 7.395 2	n/a (WT)
Check 3	7.395 1, 7.395rev#1	n/a (WT)

Figure 5.6: Location of walk and check fragments and the enzymes and primers used to generate those fragments in the genomic map of mutant YB7.395. The highlighted *Bam*HI restriction site indicates the pAN7-1 integration site. DNA abbreviations: (M) – mutant DNA; (WT) wild type or parental DNA; (n/a) – no restriction enzyme used. Restriction enzyme site abbreviations: B – *Bam*HI; E – *Eco*RV. Restriction enzyme sites *Pvu*II and *Ssp*I present at the respective terminal fragment ends of walk-3 and walk-5 are not marked in Fig 5.6.

5.3.2.4 Mutant YB7.412

Figure 5.7 shows the PCR fragments amplified to identify the sequence surrounding the integration site in the genome of mutant YB7.412. The initial fragment, walk-1, was amplified by primers PlaR2 and PlaR2.1 from the PlaR2.1 primer to an *Eco*RV site (3246 bp). Primer pair 7.412 1 and 7.412 1.1 were designed from the walk-1 fragment and used to amplify the walk-2 fragment that spanned the distance from the nested 7.412 1.1 primer to the *Ssp*I restriction site (4483 bp). PCR walking was then continued with the design of primers 7.412 2 and 7.412 2.1. The nested primer 7.412 2.1 amplified the walk-3 fragment to an *Eco*RV site (Fig 5.7).



Fragment	Primers	Digest(DNA)
Walk 1	PlaR2, PlaR2.1	*Eco*RV(M)
Walk 2	7.412 1, 7.412 1.1	*Ssp*I(M)
Walk 3	7.412 2, 7.412 2.1	*Eco*RV(M)
Walk 4	7.412 3, 7.412 3.1	*Sca*I(WT)
Walk 5	7.412 3, 7.412 3.1	*Pvu*II(WT)
Walk 6	7.412 5, 7.412 5.1	*Swa*I(WT)
Walk 7	7.412 6, 7.412 6.1	*Eco*RV(M)
Check 1	7.412check#2, 7.412check#3	n/a (WT)
Check 2	7.412check#2, 7.412check#5	n/a (WT)
Check 3	7.412check#4, 7.412check#5	n/a (WT)
Check 4	7.412sbffwd, 7.412check#5	n/a (WT)
Check 5	7.412a, 7.412check#3	n/a (WT)

Figure 5.7: Location of walk and check fragments and the enzymes and primers used to generate those fragments in the genomic map of mutant YB7.412. The *Bam*HI restriction site highlighted indicates the pAN7-1 integration site. DNA abbreviations: (M) – mutant DNA; (WT) wild type or parental DNA; (n/a) – no restriction enzyme used. Restriction site abbreviations: B – *Bam*HI; E – *Eco*RV; P – *Pvu*II; Sc – *Sca*I; Swa – *Swa*I; S – *Ssp*I. Cleavage sites for the restriction enzyme *Eco*RV present at the terminal fragment ends of walk-3 and walk-7 are not marked in Fig 5.7.

The walking primers PlaR1 and PlaR1.1 failed to produce a fragment and consequently new primers, 7.412 3 and 7.412 3.1, were designed and oriented such that extension would occur in the direction of the *Bam*HI site where integration occurred. Strain 5 genomic DNA was used as template for the PCR walk and the nested primer 7.412 3.1 amplified the walk-4 and -5 fragments to the *Sca*I (1878) and *Pvu*II (1286) restriction sites, respectively. The walk-5 fragment was used to design primers 7.412 5 and 7.412 5.1 that amplified the walk-6 fragment. The walk-6 fragment, that was amplified twice in different walking reactions, was the product of primer 7.412 5.1

amplification to the *Swa*I site (483 bp). One more pair of nested primers was designed from the walk-6 fragment, 7.412 6 and 7.412 6.1, and used to amplify two different walk-7 fragments, approximately 600 bp and 2 kb in length. These fragments were both products of the 7.412 6.1 nested primer and terminated at *Eco*RV restriction sites. Neither fragment was sequenced entirely because it was considered that enough sequence data on the flank of this mutant had been collected.

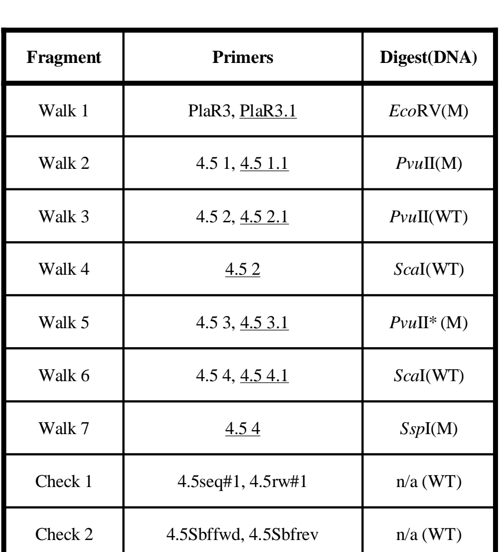
In total, 5 PCR fragments were generated and sequenced to prove the overlap between walking fragments. The two most significant PCR fragments used to confirm walking fragment overlap were the check-4 and -5 fragments. The check-4 fragment, amplified with primers 7.412sbffwd and 7.412check#5, verified the overlap of the walk-4 and -5 fragments with the walk-1 fragment. Furthermore, this check fragment also confirmed the overlap of walk-1 with -2 and walk -2 with -3. The other significant PCR fragment, check-5, amplified with the primers 7.412a and 7.412check#3, verified the overlap between the walk-5 and walk-6 fragments. The walk-7 fragment overlap with walk-6 was not independently verified because 292 bp of overlap already existed between the fragments.

5.3.2.5 Mutant YH4.5

The location and length of fragments amplified by either general PCR (Check fragments) or PCR walking (Walk fragments) used in the isolation of genomic sequence surrounding the integration site of mutant YH4.5 is shown below (Fig 5.8). The initial PCR walk from the pAN7-1 integration site into the fungal genome was produced by amplification with the nested walking primer PlaR3.1 to the *Eco*RV site located at 3664 bp. The primer pair 4.5 1 and 4.5 1.1 were then designed from this fragment and used to amplify the walk-2 fragment originating from the nested primer 4.5 1.1 to a *Pvu*II restriction site.

The PlaR4 and PlaR4.1 primers from the pAN7-1 integration failed to produce a walking fragment. Consequently, the primers 4.5 2 and 4.5 2.1 were designed from the walk-1 fragment with 3' extension directed toward the *Hind*III restriction site where integration occurred. Two products were amplified on parental DNA with this primer pair. Walk-3, extended from the nested primer 4.5 2.1 to the *Pvu*II restriction site (1805 bp). The second fragment, walk-4, was amplified by 4.5 2 and terminated at the *Sca*I restriction site (1604bp). The walk-4 fragment was then used to design the primer pair 4.5 3 and 4.5 3.1 that amplified the walk-5 fragment from a *Pvu*II digested

DNA walking stock. The walk-5 fragment amplified from the primer 4.5 3.1 terminated at a site not consistent with the *Pvu*II palindrome (CAACTG - 1436bp). The walk-5 fragment was used to design the primer pair 4.5 4 and 4.5 4.1 that amplified two fragments, walk-6 and -7. Walk-6 was produced from the nested primer 4.5 4.1 and terminated at the *Sca*I restriction site (639bp). Walk-7 was a fragment approximately 1.6 kb in length (data not shown), amplified by primer 4.5 4 from mutant DNA digested with *Ssp*I as a template.



Fragment	Primers	Digest(DNA)
Walk 1	PlaR3, PlaR3.1	*Eco*RV(M)
Walk 2	4.5 1, 4.5 1.1	*Pvu*II(M)
Walk 3	4.5 2, 4.5 2.1	*Pvu*II(WT)
Walk 4	4.5 2	*Sca*I(WT)
Walk 5	4.5 3, 4.5 3.1	*Pvu*II* (M)
Walk 6	4.5 4, 4.5 4.1	*Sca*I(WT)
Walk 7	4.5 4	*Ssp*I(M)
Check 1	4.5seq#1, 4.5rw#1	n/a (WT)
Check 2	4.5Sbffwd, 4.5Sbfrev	n/a (WT)

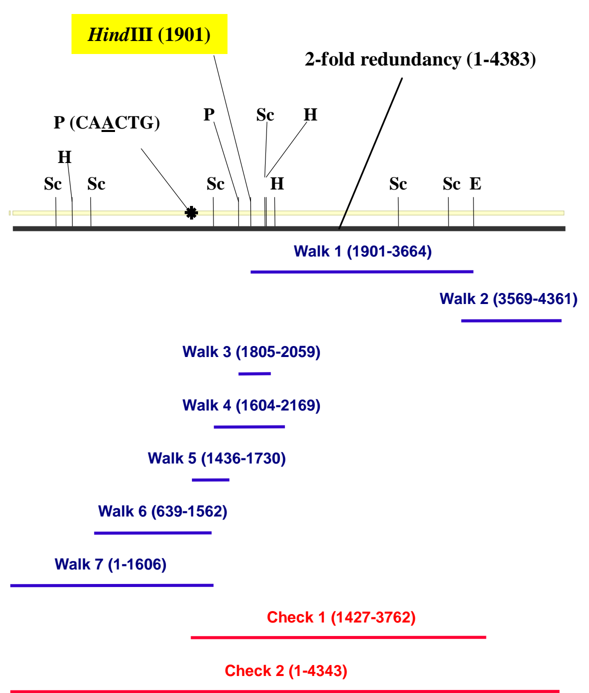


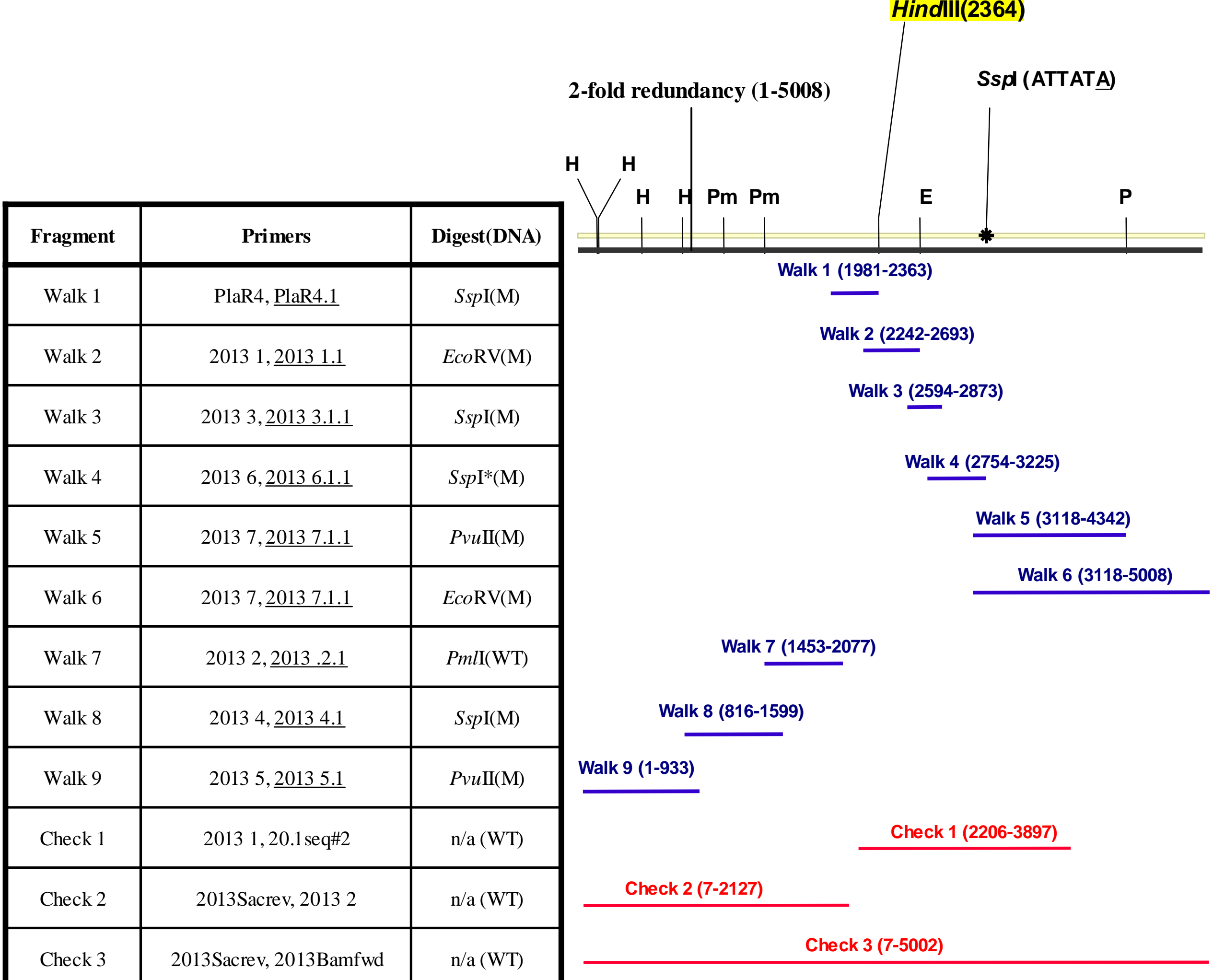
Figure 5.8: Location of walk and check fragments and the enzymes and primers used to generate those fragments in the genomic map of mutant YH4.5. The highlighted *Hind*III restriction site indicates the pAN7-1 integration site. DNA abbreviations: (M) – mutant DNA; (WT) wild type or parental DNA; (n/a) – no restriction enzyme used. Restriction site abbreviations: E – *Eco*RV; H – *Hind*III; P – *Pvu*II; Sc – *Sca*I. The *Pvu*II site marked as an asterisk varied from the normal *Pvu*II restriction palindrome by a single base pair (underlined). Cleavage sites for the restriction enzymes *Pvu*II and *Ssp*I present at the terminal fragment ends of walk-2 and walk-7 are not marked in Fig 5.8.

Two fragments, check-1 and -2, were amplified and sequenced to prove walking fragment overlap. Check-1, amplified from 4.5seq#1 and 4.5 rw#1 and check-2, amplified with 4.5SbfIrev and 4.5SbfIfwd, both show that all fragments produced by PCR walking overlapped.

5.3.2.6 Mutant LH2013

Figure 5.9 shows the location and length of fragments amplified by PCR to deduce the sequence flanking the integration site in the genome of mutant LH2013. Initially, primer PlaR4.1 amplified the walk-1 fragment from the pAN7-1 integration site from a DNA walking stock digested with the blunt end restriction enzyme *Ssp*I. Unexpectedly, at the point where adaptor ligation occurred no *Ssp*I restriction site was observed. Furthermore, the sequence at the point of adaptor ligation showed no resemblance to the *Ssp*I hexanucleotide palindrome. Primers 2013 1 and 2013 1.1 were designed from walk-1 and amplified the walk-2 fragment to an *Eco*RV site (2693). Primers 2013 3 and 2013 3.1 were designed and used to amplify the walk-3 fragment from *Ssp*I digested DNA walking stock. Once again the adaptor ligated at a site that did not resemble the *Ssp*I hexanucleotide palindrome. The walk-4 fragment, amplified from the nested primer 2013 6.1, terminated at a site that differed from the *Ssp*I palindrome, AATATT by a single base pair, AATAT<u>A</u>. The primer pair 2013 7 and 2013 7.1 were designed from the walk-3 fragment and were used to amplify two walk fragments, walk-5, that amplified to a *Pvu*II site (4343) and walk-6, that amplified to an *Eco*RV site.

The first attempts at amplifying a product on the opposite flank of the integration were unsuccessful. Consequently, primers 2013 1 and 2013 1.1 were designed from the walk-1 fragment and orientated towards the integration site. Extension from the nested primer 2013 1.1 produced the walk-7 fragment on UK7 parental DNA as a template that terminated at a *Pml*I restriction site (1453). The nested primer 2013 4.1 amplified the walk-8 fragment from the *Ssp*I digested DNA walking stock. Again the *Ssp*I palindrome was not observed at the location of adaptor ligation. This fragment was, however, used to design the 2013 5 and 2013 5.1 primers that amplified the walk-9 fragment from *Pvu*II digested walking stock.



Fragment	Primers	Digest(DNA)
Walk 1	PlaR4, PlaR4.1	*Ssp*I(M)
Walk 2	2013 1, 2013 1.1	*Eco*RV(M)
Walk 3	2013 3, 2013 3.1.1	*Ssp*I(M)
Walk 4	2013 6, 2013 6.1.1	*Ssp*I*(M)
Walk 5	2013 7, 2013 7.1.1	*Pvu*II(M)
Walk 6	2013 7, 2013 7.1.1	*Eco*RV(M)
Walk 7	2013 2, 2013 .2.1	*Pml*I(WT)
Walk 8	2013 4, 2013 4.1	*Ssp*I(M)
Walk 9	2013 5, 2013 5.1	*Pvu*II(M)
Check 1	2013 1, 20.1seq#2	n/a (WT)
Check 2	2013Sacrev, 2013 2	n/a (WT)
Check 3	2013Sacrev, 2013Bamfwd	n/a (WT)

Figure 5.9: Location of walk and check fragments and the enzymes and primers used to generate those fragments in the genomic map of mutant LH2013. The *Hind*III restriction site highlighted indicates the pAN7-1 integration site. DNA abbreviations: (M) – mutant DNA; (WT) wild type or parental DNA; (n/a) – no restriction enzyme used. Restriction enzyme site abbreviations: B – *Bam*HI; E – *Eco*RV; P – *Pvu*II; Sc – *Sca*I; S – *Ssp*I. The *Ssp*I site marked as an asterisk varied from the normal *Ssp*I palindrome by a single base pair (underlined) suggesting star activity (see 5.3.3). Cleavage sites for the restriction enzymes *Pvu*II and *Ssp*I present at the terminal fragment ends of walk-2 and walk-7 are not marked in Fig 5.9.

Three check fragments, check-1, -2 and -3 were used to verify fragment overlap. Check-1 used primers 2013 1 and 20.1seq#2 to produce a fragment that overlapped the walk-1, -2, -3, -4, -5 and -6 fragments. Check-2, amplified with 2013Sacrev and 2013 2 spanned the remaining walk fragments, -7, -8 and -9. The final fragment, check-3, was amplified with 2013Sacrev and 2013Bamfwd and showed that all walk and check fragments originated from one genomic location.

5.3.3 *Pvu*II and *Ssp*I Digestion and Star Activity

Star activity, i.e., relaxation of enzyme specificity, has previously been reported for several restriction enzymes including *Pvu*II and *Ssp*I (Nasri and Thomas, 1987; Nath and Azzolina, 1981; New England Biolabs, unpublished). Two walk fragments amplified from DNA walking stocks of different mutants, both digested with restriction enzyme *Pvu*II, terminated at sites that were not the canonical *Pvu*II hexanucleotide palindrome, CAGCTG. Nasri and Thomas (1987) observed that the restriction endonuclease *Pvu*II decreases its substrate specificity in the presence of organic solvents and cleaves the sequence CAGCTG with any nucleotide substitution at any one of the six positions within the sequence. While there were no organic solvents present during restriction enzyme digestion, the reaction mix was purified with a phenol/chloroform extraction. Thus during phenol/chloroform extraction, endonuclease activity may have continued reducing the specificity of *Pvu*II to recognise the hexanucleotide sequences CACCTG in mutant YB4.44 and CAACTG in mutant YH4.5.

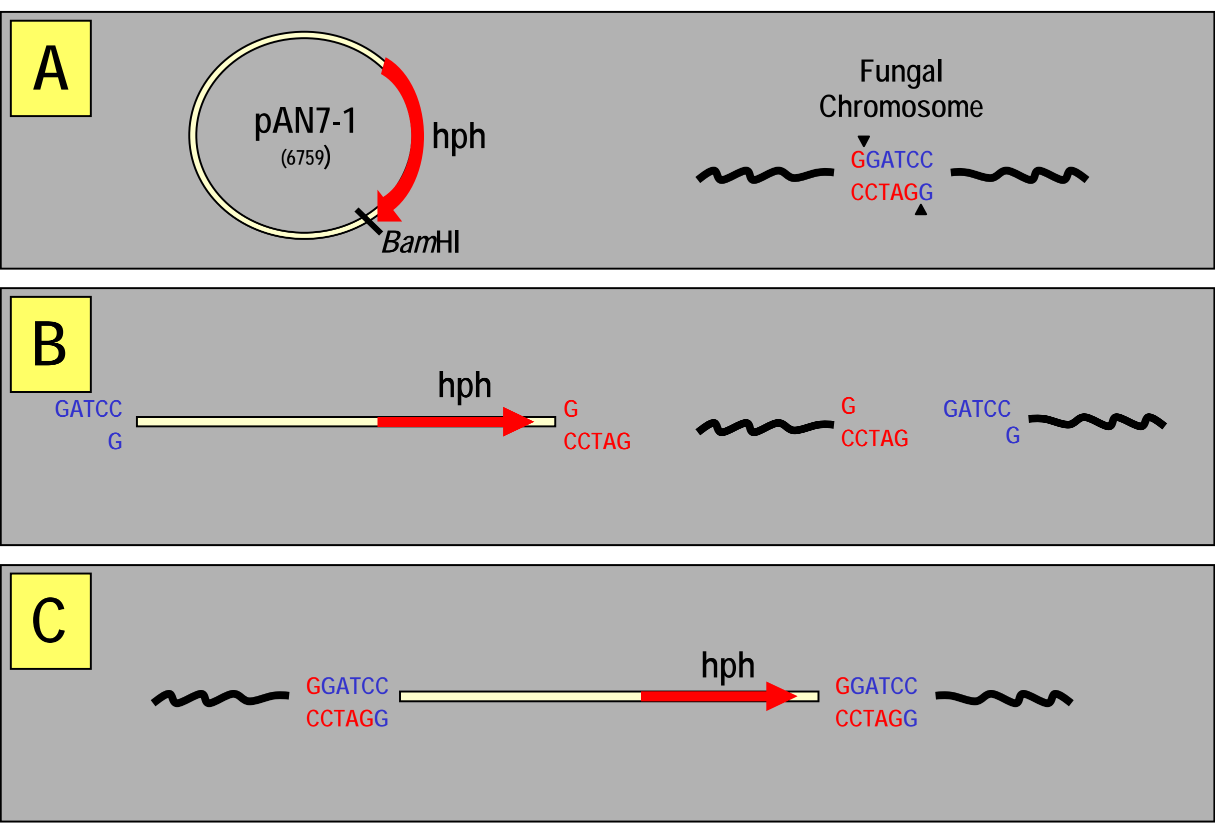
The DNA walking stock of mutant LH2013 digested with *Ssp*I produced some unexpected results. On three occasions the adaptor duplex ligated where no *Ssp*I restriction enzyme site existed. The terminal cleavage site of the three walk fragments, walk-1, -3 and -7 showed no similarity to any commercially available blunt end restriction enzymes. Furthermore, no sequence similarities to the *Ssp*I palindrome were observed at or near the point where adaptor duplex ligation occurred in any of the three walk fragments. This phenomenon occurred only in LH2013 DNA walking stock digested with *Ssp*I and, therefore, is likely to be the result of a contaminant or handling issue that randomly created blunt end sites and fragments suitable for adaptor ligation. The walk-4 fragment amplified from the LH2013 *Ssp*I walking stock also terminated at a location that did not exactly match the *Ssp*I hexanucleotide palindrome. However, in this case the observed hexanucleotide sequence only deviated by a single base pair, AATAT<u>A</u> compared to AATATT, that

could have been a result of star activity which has previously been observed in the enzyme *Ssp*I (Nath and Azzolina, 1981; New England Biolabs, unpublished).

5.3.4 Structure of the REMI Junctions

Theoretically, a REMI integration event should occur in the following way: cleavage of plasmid and fungal chromosomal DNA by restriction enzyme, integration of the linearised plasmid into the chromosomal DNA at an open, cleaved restriction site and ligation of the compatible ends of the non-homologous DNA molecules. Depending on the junction sequences created between fungal genomic and plasmid DNA after integration, two types of REMI integrations occur. The first, a conservative REMI event, occurs if no DNA sequence is altered during the transformation process and the original restriction sites are reproduced at the junctions of plasmid and genomic DNA (Fig 5.10). The second type of integration is considered non-conservative, where only one or neither of the original restriction sites is conserved.

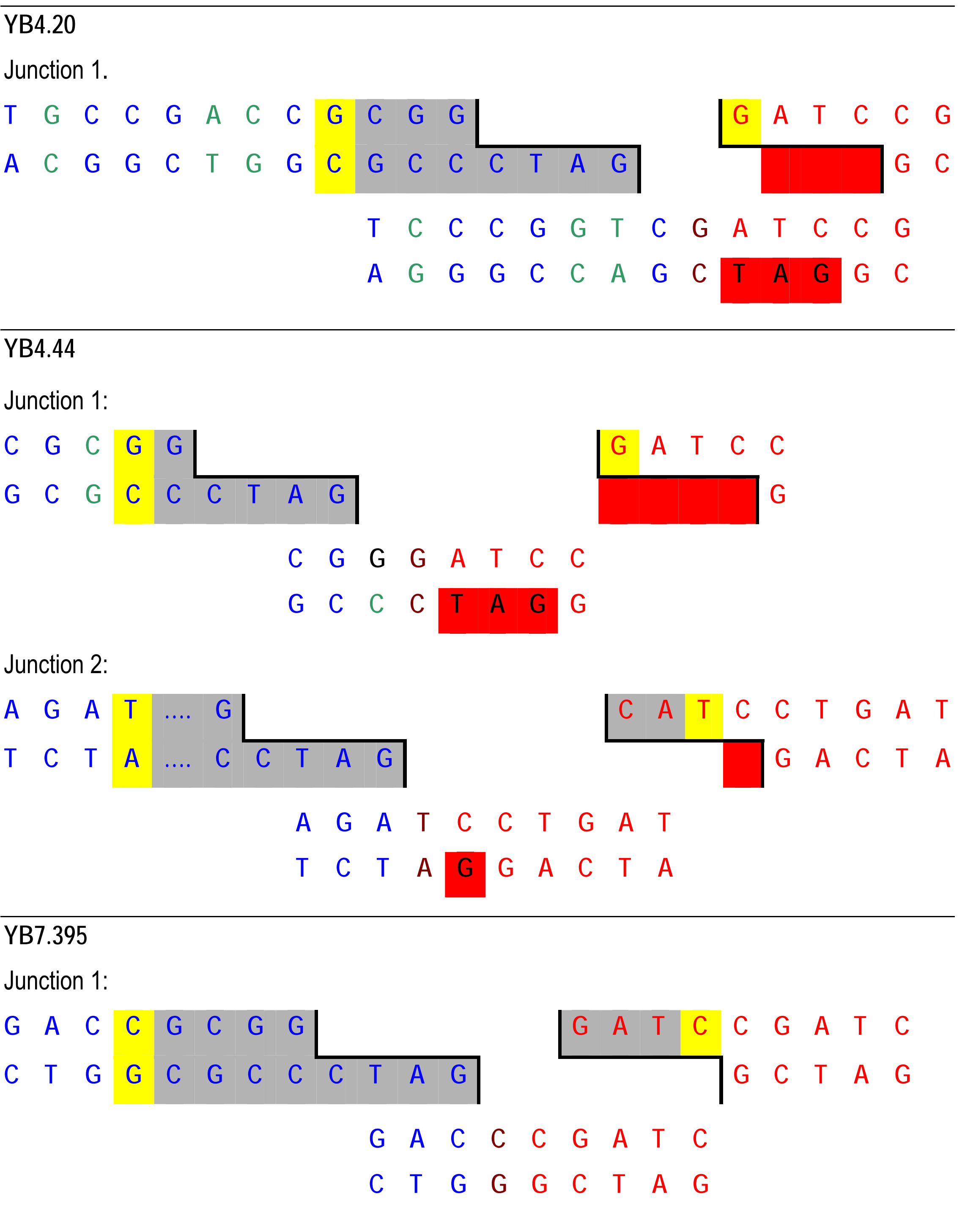
To further investigate the REMI mechanism in *R. secalis*, most of the junction sites of the six selected mutants were sequenced (Fig 5.11). In each REMI integration, two junction sites are created as shown diagrammatically in Figs 5.1 and 5.10. All junction sequences were obtained by sequencing PCR walk fragments. In most cases these fragments were generated from primers located within the integrated plasmid. However, where this strategy failed, primers located on fungal chromosomal DNA with extension directed toward the plasmid integration were used to amplify fragments containing the second integration junction from fungal mutant DNA walking stocks. A diagrammatic representation of the fragments that contained junction sequences is presented later (Fig 5.12 and Fig 5.13).



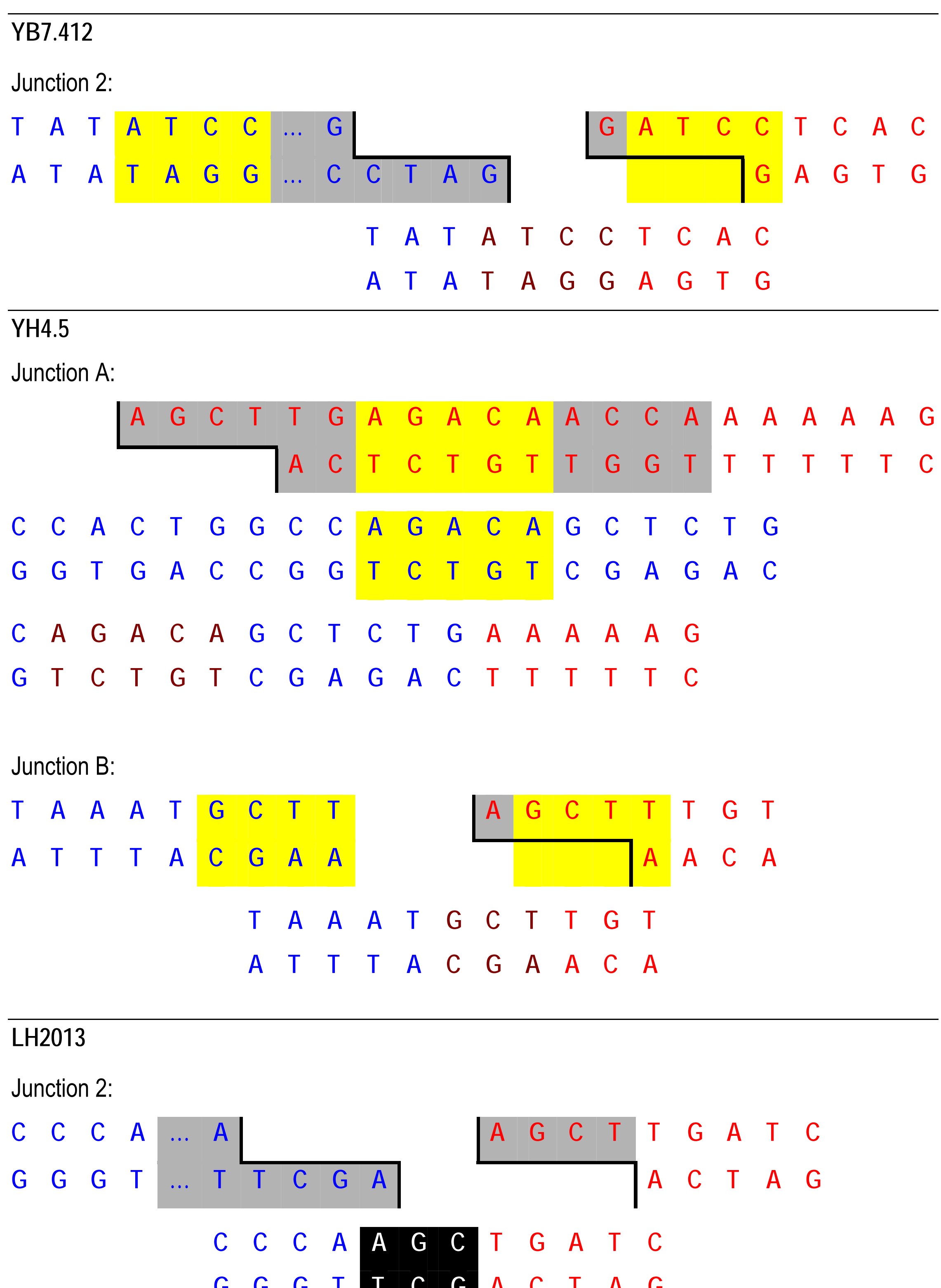
D. The *Hind*III palindrome:

A | A G C T T
T T C G A | A

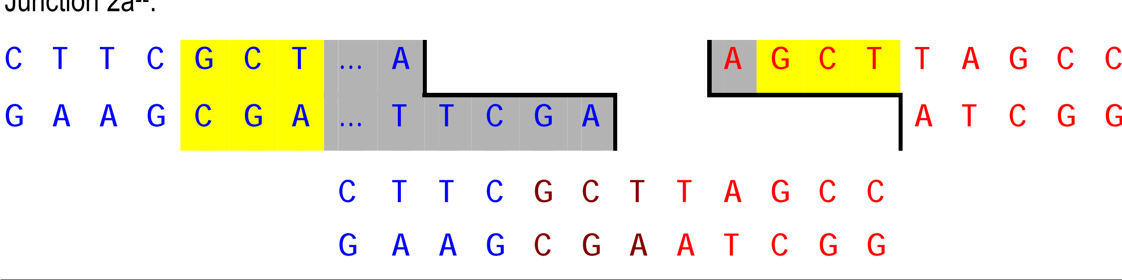
Figure 5.10: Restriction sites during REMI transformation. A. Location of the *Bam*HI restriction sites in both the transformation vector pAN7-1 and the fungal chromosome. B. After cleavage with the restriction enzyme *Bam*HI, both the linearised plasmid and the fungal chromosome have 4-bp 5' overhangs. C. The 5' overhangs of the two molecules associate and the fungal DNA repair machinery forms covalent bonds. Once the plasmid DNA has integrated into the fungal chromosome, a *Bam*HI restriction enzyme site exists at both ends of the plasmid DNA. D. If the restriction enzyme *Hind*III is used in REMI transformation, the equivalent process occurs with the exception that the double stranded palindrome sequence differs from than of *Bam*HI. Abbreviation: hph - hygromycin resistance gene.



YB4.20
Junction 1.
T G C C G A C C G C G G
A C G G C T G G C G C C C T A G
G A T C C G
G C
T C C C G G T C G A T C C G
A G G G C C A G C T A G G C
YB4.44
Junction 1:
C G C G G
G C G C C C T A G
G A T C C
G
C G G G A T C C
G C C C T A G G
Junction 2:
A G A T .... G
T C T A .... C C T A G
C A T C C T G A T
G A C T A
A G A T C C T G A T
T C T A G G A C T A
YB7.395
Junction 1:
G A C C G C G G
C T G G C G C C C T A G
G A T C C G A T C
G C T A G
G A C C C G A T C
C T G G G C T A G


YB7.412
Junction 2:
T A T A T C C ... G
A T A T A G G ... C C T A G
G A T C C T C A C
G A G T G
T A T A T C C T C A C
A T A T A G G A G T G
YH4.5
Junction A:
A G C T T G A G A C A A C C A A A A A A G
A C T C T G T T G G T T T T T T C
C C A C T G G C C A G A C A G C T C T G
G G T G A C C G G T C T G T C G A G A C
C A G A C A G C T C T G A A A A A G
G T C T G T C G A G A C T T T T T C
Junction B:
T A A A T G C T T
A T T T A C G A A
A G C T T T G T
A A C A
T A A A T G C T T G T
A T T T A C G A A C A
LH2013
Junction 2:
C C C A ... A
G G G T ... T T C G A
A G C T T G A T C
A C T A G
C C C A A G C T G A T C
G G G T T C G A C T A G

Junction 2a$^{\Omega}$:



Legend:

G Blue coloured base pairs represent plasmid DNA sequence

A Red coloured base pairs represent genomic DNA sequence

G Green coloured base pairs represent base pairs that change during the integration process

A Crimson coloured base pairs are common to both genomic and plasmid sequences

Grey shading represents base pairs deleted in the ligation process

.... Dotted line with grey shading represents a stretch of base pairs deleted from the plasmid DNA prior to or during the ligation process

Red shading represents base pairs that have been filled in or repaired

Yellow shading represents base pairs common to both genomic and plasmid sequences

A Black shading combined with white base pairs represent the inclusion of base pairs from unknown origin

Bold lines represent the 3' overhang created by the restriction enzymes *Bam*HI or *Hind*III

Figure 5.11: DNA sequence of plasmid pAN7-1* and fungal genomic* DNA prior to integration and the DNA sequence of the junction site after plasmid integration. Mutants were created with the *Bam*HI restriction enzyme with the exception of mutants YH4.5 and LH2013 where *Hind*III was used. Where a stretch of base pairs was deleted from the plasmid the number of deleted base pairs is discussed, with respect to the individual mutant junction, in the text.

*Note: with the exception of YH4.5, the pAN7-1 and genomic sequences presented in this figure have been shown after the restriction enzyme activity assumed to facilitate integration. The location of pAN7-1 cleavage in mutant YH4.5 is discussed below.

Junction 2a$^{\Omega}$: both junctions in mutant LH2013 begin at the same end of the linearised plasmid.

Only one of the two plasmid-chromosome junctions was sequenced in mutant YB4.20 (Fig 5.11). The sequence observed at junction 1 was consistent with a non-conservative REMI integration and both genomic and plasmid DNA ends were processed. Processing of the plasmid end included the deletion of at least 7 bp from the pAN7-1 sequence and the conversion of 3 bp. The genomic DNA end has at least 3 bp of the 5' *Bam*HI restriction enzyme overhang filled in. At the precise point where the two sequences meet there is a 1 bp microhomology.

Sequencing data were collected for both junction sites of the pAN7-1 integration in mutant YB4.44 (Fig 5.11) and showed that genomic and plasmid DNA 5' overhangs had been processed. At junction 1, at least 5 bp were degraded or deleted from the plasmid and 1 bp converted adjacent to the junction site. The genomic 5' overhang had at least 3 bp filled in and a one base microhomology exists between the two sequences.

Sequencing at junction 2 showed processing of plasmid and genomic DNA ends had occurred. The plasmid molecule end that formed junction 2 had at least 41 bp deleted. The genomic sequence appears to have 2 bp deleted from the terminal end and at least 3 bp of the 5' overhang filled in. Finally, a 1 bp microhomology exists at the junction point.

Sequencing of the junction 1 site of mutant YB7.395 showed the plasmid and genomic DNA ends were processed after *Bam*HI cleavage (Fig 5.11) with at least 8 bp deleted from the plasmid and at least 3 bp deleted or degraded from the genomic DNA. At the location where the two sequences meet there is a 1 bp microhomology.

The second junction site, junction U, of mutant YB7.395 showed a fully reformed *Bam*HI site (data not shown). However, at junction U no sequence homology to pAN7-1 was observed. In this case junction U sequence information was deduced from a PCR walking fragment generated using a primer directed toward the integration site, 7.395 check#1, and mutant YB7.395 DNA as template. A fragment 774 bp in length was amplified and sequenced. Sequence comparisons showed 138 bp were 100% identical to mutant YB7.395 genomic sequence up to the *Bam*HI site that was cleaved to create the integration site. However, the remainder of the fragment (636 bp) showed no sequence similarity to the pAN7-1 sequence or any known *R. secalis* genomic sequence (Fig 5.13).

In the mutant YB7.412 no sequence at junction 1 was generated. The sequence of the junction 2 site showed at least 45 bp were deleted from the pAN7-1 plasmid end (Fig 5.11).

Furthermore, at least 1 bp was deleted from the fungal genomic sequence leaving a 4 bp microhomology at the junction point between fungal and plasmid DNA integration.

The junction sequences in mutant YH4.5 showed the plasmid integrated into a *Hind*III site in the fungal genome. However, the plasmid sequences observed at the junctions are inconsistent with a *Hind*III linearised plasmid molecule in their location and orientation. The sequence of the two junction sites observed is represented in Fig 5.11.

At junction A, a walking fragment amplified using YH4.5 mutant DNA as a template and primers directed towards the integration site, 4.5check#1 and 4.5rw#1, showed 15 bp were deleted from the genomic sequence at the point of *Hind*III cleavage. The plasmid sequence observed at junction A is located a considerable distance from the *Hind*III site on the plasmid. To create the observed plasmid sequence at junction A, more than 2.6 kb would have been degraded or deleted from the end of a *Hind*III linearised pAN7-1 molecule. A 5 bp microhomology was present 6 bp from the junction point and was retained in the plasmid sequence after integration was completed.

Fungal genomic sequence observed at junction B had 1 bp degraded from the terminal end of the 5' *Hind*III overhang. The plasmid sequence observed in junction B is located 574 bp from the *Hind*III site in pAN7-1. Furthermore, the sequenced walk-1 fragment showed an intact *Hind*III site in the sequence. Finally, a 4 bp microhomology exists at the junction point between genomic and plasmid sequences (Fig 5.11).

The two junction sites in the DNA from mutant LH2013 show sequence from the same ends of the linearised plasmid molecule. The sequence observed at junction 2 of the mutant LH2013 indicated that 36 bp of the plasmid sequence and 4 bp of the fungal genomic sequence were degraded prior to ligation. Furthermore, at the junction site a 3 bp microhomology was present.

Junction 2a was sequenced from a reverse walk amplified with the 2013check#2 primer. The sequence showed 105 bp were degraded from the plasmid sequence and 1 bp degraded from the genomic sequence. At the junction point where the two non-homologous sequences met, 3 bp of microhomology was observed.

5.3.5 Mutant Integration Structures

In addition to Southern data, the availability of sequence information at junction sites enabled a more accurate determination of mutant integration structures and in many cases DNA sequence flanking the integration sites confirmed fragment sizes observed in Southern analysis (Fig 5.2). Mutants with both junctions sequenced will be discussed separately from those with only one junction completed.

5.3.5.1 Mutants with Sequence Information at both Integration Junctions

For three mutants, YB4.44, YH4.5 and LH2013, both integration junctions were sequenced. In combination with Southern data a diagrammatic representation of integration was deduced (Fig 5.12).

Mutant YB4.44 contains a single integration yielding fragments of >9383 bp when digested with *Eco*RV and of >2856bp and >8006 bp when cleaved with *Nco*I. This is expected of a single-copy, single-site integration. Similarly, mutant YH4.5 displayed a Southern hybridisation banding pattern consistent with a simple, single integration. However, in contrast to mutant YB4.44, junction sequences revealed that this was not the case. Although sequencing shows that integration occurred into a single genomic *Hind*III site, the plasmid sequence was not consistent with a simple linearization with *Hind*III. In fact, cleavage at one site of the pAN7-1 molecule could not have been responsible for the two observed junction sequences. Given that both of the genomic-plasmid DNA junctions are oriented in the same direction on the pAN7-1 plasmid (Fig 5.12), two pAN7-1 plasmids, or truncated versions of the plasmid, must have integrated into the fungal genome at the same site in opposite orientation. Digestion with *Nco*I and hybridisation with probe 1 produced only two fragments (Fig 5.2) and, therefore, one *Nco*I site from the two integrating pAN7-1 plasmids was removed. Sequencing from the junction B site showed an *Nco*I site in the flanking genomic DNA that, combined with an *Nco*I site in pAN7-1 [2] was responsible for the fragment 3160 bp in length observed by Southern analysis (Fig 5.2, Fig 5.12). Therefore, the pAN7-1 [1] fragment (Fig 5.12) must be truncated to remove one *Nco*I site. The remaining length of the fragment is difficult to estimate because the position where truncation of pAN7-1 [1] occurred is difficult to ascertain without extensive sequencing or Southern analysis.

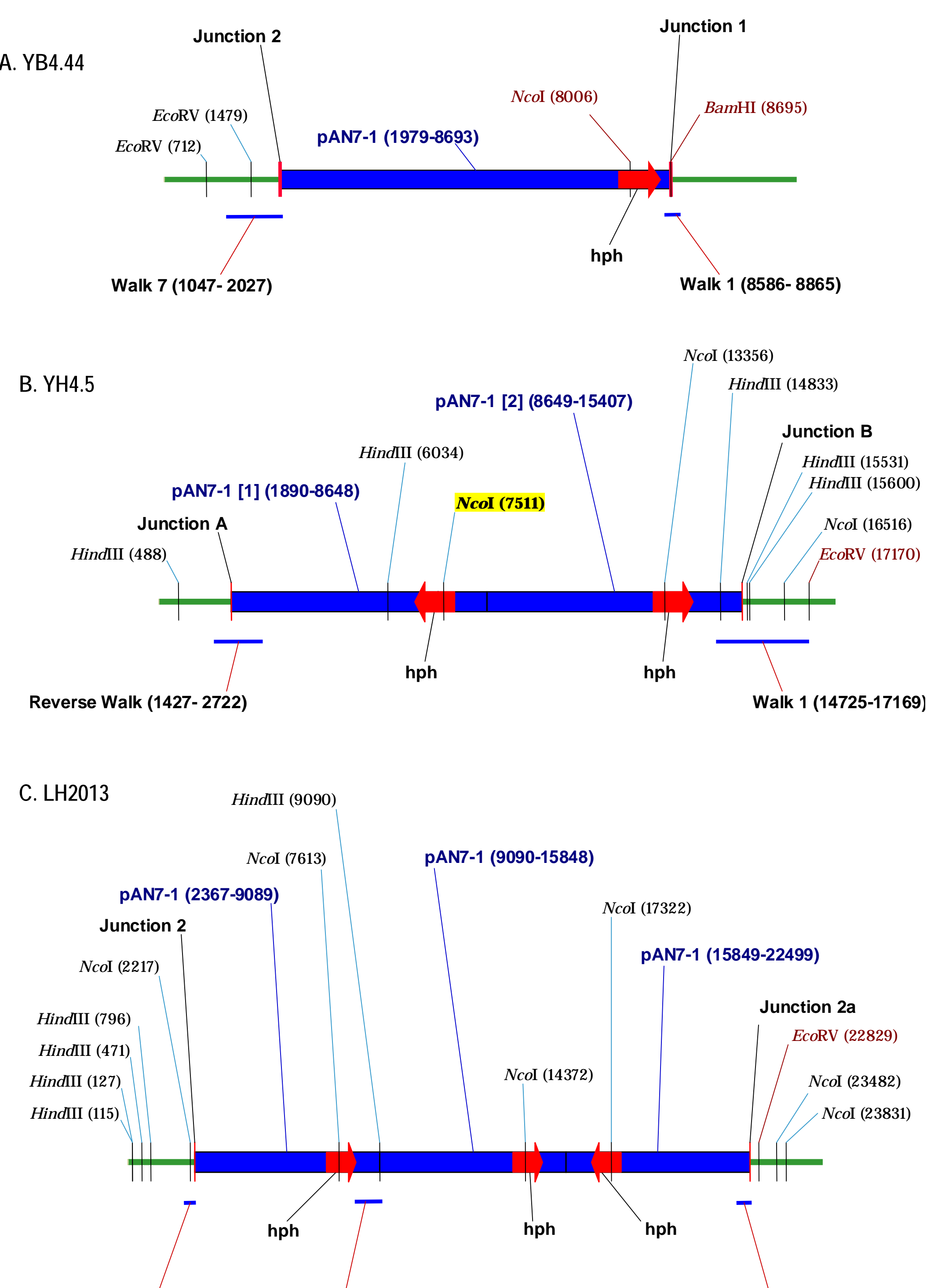


Figure 5.12: Three mutants, A. YB4.44, B. YH4.5 and C. LH2013, with both integration junctions sequenced. Integration structures were deduced from Southern results and sequencing. Regions sequenced are identified as walk fragments. Abbreviation: hph - hygromycin resistance gene.

Mutant LH2013 also shows a complex integration pattern. Southern analysis of this mutant with enzymes *Eco*RV and *Nco*I produced one and four fragments, respectively. Considering a single *Eco*RV band indicates integration at a single genomic location, four fragments observed upon *Nco*I digestion suggests that three pAN7-1 molecules had integrated in tandem array. Junction sequences support multiple integrations indicating that at least two integrations had occurred in a head-to-tail, tail-to-head tandem array. Furthermore, a PCR walk fragment generated with PlaR4.1 revealed sequence information indicating a head-to-tail junction between two pAN7-1 molecules (Fig 5.12). From this information it can be concluded that at least three pAN7-1 molecules are present in the *Hind*III integration site of mutant LH2013. The diagrammatic representation of the integration structure (Fig 5.12) has been based on the assumption that three plasmid molecules integrated and the one unsequenced junction between 2 plasmid molecules was a conservative REMI junction. Using these assumptions and the integrations flanking sequence, the fragment sizes 5358 bp, 6055 bp, 6759 bp, and 2950 bp are expected and observed in Southern hybridisation using the enzyme *Nco*I and probe 1.

5.3.4.2 Mutants with Sequence Information at One Plasmid-DNA Integration Junction

Although mutants YB4.20, YB7.395 and YB7.412 had only one of two integration junctions sequenced, likely integration structures have been deduced based on Southern and sequence data available. The structure of the YB4.20 integration was deduced from the sequence of the junction site and flanking genomic DNA. Based on the sequenced junction 1 site, a fragment 2946 bp in length was expected upon *Nco*I cleavage and indeed observed on the autoradiograph (Fig 5.2). If a conservative REMI junction is presumed at the other junction, a second *Nco*I fragment of >9036 would be expected, which was also observed on the autoradiograph.

From mutant YB7.395 genomic flanking sequence was initially obtained by PCR walking with primer PlaR1.1. This sequence at junction 1 was consistent with the other mutants, showing DNA ligation between pAN7-1 and fungal genomic DNA (Fig 5.11, Fig 5.13). In contrast, sequence information at the other junction (junction U) revealed that the fungal genomic DNA was not ligated to the linearised pAN7-1 molecule and was, in fact, ligated to DNA that matched neither plasmid nor known *R. secalis* genomic DNA (Fig 5.13). No further sequencing was performed and, consequently, no sequence connecting fungal and plasmid DNA was obtained and the structure of

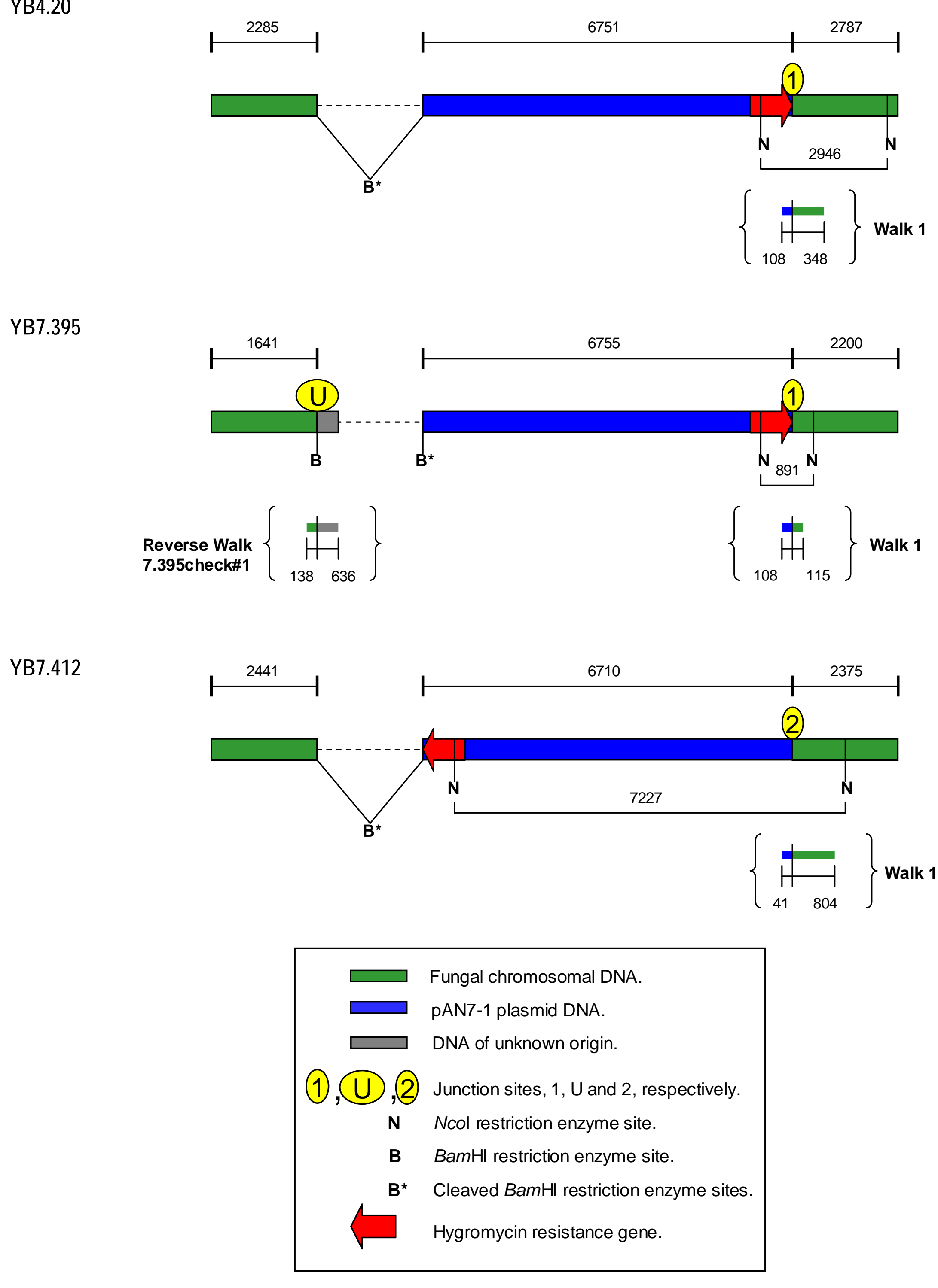


Figure 5.13: Three mutants, YB4.20, YB7.395 and YB7.412, with one plasmid-genomic DNA integration junction sequenced. The dotted line present between DNA types indicates an unknown junction. Integration structures were deduced from Southern results and sequencing. Regions sequenced are identified as walk fragments.

this integration can not be determined. However, based on the sequence obtained from the junction 1 site of mutant YB7.395, two fragments produced following *Nco*I cleavage should be 891 bp and >6068 bp in length. These fragments are observed in Southern analysis of this mutant (Fig 5.2).

Of the final mutant, YB7.412, only one of the two junction sites, junction 2, was sequenced. For the purpose of representing the integration diagrammatically the pAN7-1 linearised plasmid has been included. Based on the *Nco*I site present in pAN7-1 and the genomic DNA flanking junction 2, a fragment of 7227 bp was expected upon cleavage with this enzyme. Furthermore, if a conservative REMI integration at junction 1 is assumed a second *Nco*I fragment of >3128 bp and an *Eco*RV fragment of >9955 bp in length were to be expected. All these fragments were found by Southern analysis (Fig 5.2).

5.4 Discussion

5.4.1 Southern and Sequencing Results – Random Re-assortment of the Fungal Genome

Southern hybridisations showed that in all analysed mutants plasmid integration occurred at a single site in the fungal genome. However, the mechanism of integration often turned out to be complex and evidence of both deletions and insertions occurred at the integration site. The observed deletion in mutant YB4.44 and insertion in mutant YB7.395 suggest that genomic rearrangements at sites unlinked to the molecular tag are likely to have occurred during these mutant's REMI transformations.

*Bam*HI cleavage in mutant YB4.44 deleted 644 bp of genomic sequence between two restriction sites (Fig 5.5) suggesting that restriction enzyme activity could cause deletions in other locations of the genome that are unlinked to the molecular tag. However, sequencing mutant DNA during PCR walking showed that predicting the occurrence of deletions between closely linked restriction sites would be difficult. In mutant YB4.20, cleavage occurred at only one of two *Bam*HI sites only 8 bp apart (Fig 5.4), no cleavage was observed between three *Hind*III restriction sites located within 193 bp of each other in YH4.5 (Fig 5.8) and, similarly, in LH2013 where 3 *Hind*III sites were present in a 669 bp stretch of DNA (Fig 5.9).

Further evidence of genomic re-assortment was exhibited by mutant YB7.395 at junction U (Fig 5.13). In this case, sequencing showed fungal DNA that was expected to flank the pAN7-1 integration was unexpectedly ligated to DNA of unknown origin, presumably from another part of the genome. This can be explained either by an extraneous chromosomal fragment inserting simultaneously with the pAN7-1 plasmid into the *Bam*HI integration site of mutant YB7.395, or that the genomic material at junction U was excised and became a chromosomal fragment that integrated at another genomic location. Although extensive sequencing or Southern analysis could resolve the structure of this mutant, the unusual integration structure combined with further sequence analysis presented in the following chapter revealed that YB7.395 was not a good candidate for the identification of fungal pathogenicity genes.

The observed integration structure in mutant YH4.5 presents a weakness in the interpretation of Southern (*cf.* chapter 3). While mutant YH4.5 produced the fragment pattern expected for a simple single-copy, single-site integration, sequencing suggested the presence of two copies of the pAN7-1 plasmid, or truncations thereof. Hence, the observed restriction fragment patterns indicative of a simple, single integration appears to have been misleading.

When digested with *Nco*I and hybridised with probe 1, DNA from mutant CH2034 produced 10 fragments. Possible explanations for this result include partial digestion of mutant DNA during Southern analysis and a number of incompletely digested fragments were observed on the autoradiograph. Alternately, considering unexpected integration structures such as that of mutant YH4.5, a number of unpredicted cleavage sites may have produced the surprising banding pattern in mutant CH2034.

5.4.2 REMI, Integration Junctions and Non-homologous End-Joining

In total, 10 out of a possible 12 integration junctions were sequenced and all were non-conservative. This result is not consistent with other REMI studies, where a proportion of conservative integrations were always observed. REMI studies performed in *Magnaporthe grisea* (Shi *et al.*, 1995), *Ustilago maydis* (Bolker *et al.*, 1995), *Penicillium paxilli* (Itoh and Scott, 1997), *Aspergillus nidulans* (Sanchez, *et al.*, 1998) and *Gibberella fujikuroi* (Linnemannstons *et al.*, 1999) all produced varying proportions of conservative integrations with non-homologous transforming DNA. However, it should be noted that only 6 mutants were examined in this study and of those only 4 mutants have had both junction sites sequenced. In the case of *G. fujikuroi* only 2 of 46

mutants were considered as true, or conservative, REMI events (Linnemannstons *et al.*, 1999). Furthermore, the REMI studies mentioned above used Southern hybridisation to determine the number of conservative and non-conservative REMI events. However, junction 1 of mutant YB4.44 shows that a *Bam*HI restriction site can be recreated without a conservative REMI integration event occurring. Sequencing showed that at least 5 bp were deleted from the plasmid end and one base pair converted within the plasmid sequence during the integration event at this junction. These events would not be detected by Southern analysis. Therefore, without sequencing some inaccuracy in calculating conservative REMI events may exist in previous studies.

Junction U of mutant YB7.395, junction A of mutant YH4.5 and junction 2 of mutant LH2013 contain integration junctions likely to have been created by complex mechanisms and, consequently, will be discussed lastly in this section. The remaining junctions appear to be a result of simpler mechanisms. Non-conservative integrations are the result of molecule end processing prior to ligation. Hence, at all of the observed junction sites in this subset of *R. secalis* mutants some form of processing occurred. Common elements exist with respect to processing and the observed junction site. Firstly, degradation of the molecule ends was consistent in mutant integrations, and degradation of the fungal chromosomal molecule was less significant than the degradation of the plasmid molecule. In general, the 5' overhang on the genomic molecules was the site of degradation. Conversely, plasmid molecules were degraded more extensively, and often, into the double stranded DNA. This suggests that cleaved fungal chromosomes were better protected to exonuclease activity than the integrating plasmid.

Molecule processing, in the form of degradation or deletion, consistently produced junction sites with microhomologies between plasmid and genomic DNA sequences. These microhomologies ranged from 1 bp to 4 bp and are important in the repair of double-strand breaks in eukaryotic cells (Paull and Gellert, 2000) through a process known as non-homologous end joining (NHEJ) (Manivasakam and Schiestl, 1998; see review Weterings and Chen, 2008). Furthermore, deletions directed back to regions of microhomology are commonly observed in NHEJ reactions responsible for double stranded break repairs in chromosomes (Chu, 1997). Linear plasmid DNA injected into *Xenopus* oocyte nuclei produced junctions containing deletions back to microhomologies of 1-10 bp (Grzesiuk and Carroll, 1987). Double strand breaks created by restriction enzymes electroporated into Chinese hamster cells were also deleted back to regions of 1-4 bp of microhomology (Phillips and Morgan, 1994). Paull and Gellert (2000) suggested that one

method of directed deletion and junction formation involves the *MreI* nuclease. This enzyme associates with a DNA molecule end containing a 5' overhang and uses its 3'-5' exonuclease activity on another molecule end until a corresponding 3' overhang, or cohesive end, is created. This hypothetical method of junction formation has also been proposed in *S. cerevisiae* (Manivasakam and Schiestl, 1988). In this study, the restriction enzymes *Asp*718 and *Kpn*I were used on plasmid and genomic DNA, respectively. Although both enzymes recognise the same 6-bp palindrome, the *Asp*718 enzyme produces a 4-bp 5' protruding single strand (PSS), whereas *Kpn*I produces a 4-bp 3' PSS end. Following transformation the original 6-bp palindrome was regained. To explain this result it was suggested that the *Asp*718 5' overhang was filled in and a 5'-3' exonuclease then created a complementary 3' overhang to the *Kpn*I digested genomic DNA (Manivasakam and Schiestl, 1998). These results are consistent with the sequences observed at many of the junction sites present in the six *R. secalis* mutants analysed.

Another possible method of junction formation is that degradation and end filling of the PSS creates blunt end molecules and these are ligated to form the observed junction sites. Studies performed in *Schizosaccharomyces pombe* showed that end filling occurred when incompatible PSS were used in REMI transformations (Goedecke *et al.*, 1994). However, considering that all junction sites contain microhomologies, directed deletions and 5'-3' exonuclease activity prior to ligation seem more likely.

Three mutants, YB7.395, YH4.5 and LH2013 contained more complex junctions that differed from the typical junction created by NHEJ between cleaved linearised plasmid and cleaved chromosomal DNA. Junction U was formed between fungal DNA flanking the *Bam*HI integration site of mutant YB7.395 and other extraneous DNA of an unknown origin. Interestingly, a *Bam*HI restriction site was regained at this junction without end processing of the known fungal sequence from the YB7.395 *Bam*HI integration locus (data not shown). However, because the expected fungal DNA does not share a junction with the pAN7-1 plasmid, this integration must be considered non-conservative.

Although integration in the fungal chromosome of mutant YH4.5 occurred within a *Hind*III restriction site, the pAN7-1 molecules that integrated do not appear to have been linearised by the *Hind*III restriction enzyme. The observed plasmid sequence at junction A is 2613bp from the *Hind*III site and thus it is possible that the plasmid was cleaved near the resulting junction sequence by some other process. Interestingly, no direct microhomology is observed at the

junction point of the two sequences. However, 6 bp from the junction point a 5 bp microhomology exists. The possibility of a directed deletion and 5'-3' exonuclease activity is excluded in this case because no direct microhomology exists at the junction point between the two sequences. One possibility is an unequal crossover event aided by the nearby 5 bp microhomology. A second possibility is that plasmid and genomic sequences were degraded back to their respective points and, as blunt end fragments, were joined by normal ligation processes.

Junction B of YH4.5 indicated that the *Hind*III site was intact in the plasmid sequence. Therefore, plasmid linearisation was not caused by *Hind*III activity. At the junction point between genomic and plasmid DNA a 4-bp microhomology exists. If the integrated plasmid was cleaved near this region of microhomology then directed deletion and 5'-3' exonuclease activity could have created compatible ends to facilitate integration.

The junction 2 sequence of mutant LH2013 appears to contain genetic material not consistent with the plasmid or genomic sequences at the junction site. A 3-bp sequence (AGC) was observed between the genomic and plasmid sequences at the junction point. Interestingly, this sequence is part of the overall 5' 4 bp overhang created by *Hind*III, AGCT. However, the possibility that the 3 bp sequence present at the junction site originates from this overhang would be difficult to asses.

5.4.3 REMI and *R. secalis* Mutagenesis – Concluding Remarks

REMI is essentially a method for facilitating integration of non-homologous DNA with potential increases in transformation efficiency (Riggle and Kumamoto, 1998). The junction sequences observed in this study with *R. secalis* show that the fundamental principles of REMI theory were still upheld because integration occurred in the fungal genome at sites created by the restriction enzyme. Furthermore, it appears that in most cases the same restriction enzyme digested and linearised the plasmid. However, deletions and insertions in the fungal genome were observed and this finding illustrates the most significant drawback to using restriction enzymes in transformation studies, untagged genomic rearrangements. Nevertheless, the DNA regions flanking the integration sites were sequenced in all mutants analysed and the availability of these sequences allowed an examination for the presence or absence of putative pathogenicity genes. This analysis is discussed in the following chapter.

Chapter 6 – Bioinformatic Analysis of Non-Pathogenic Mutants

6.1 Introduction

Genomic sequence flanking the pAN7-1 integration site from six non-pathogenic *R. secalis* mutants was examined for the presence of putative pathogenicity genes. The presence of a putative gene was used to identify mutants suitable for knock out studies. A combination of sequence analysis programs and database searches were used to identify putative genes.

The vast expansion and application of molecular biology and genetics to many living organisms have led to the identification of a large number of genes and gene functions that have been combined into publicly available databases. Furthermore, the development of the Basic Logical Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) has meant that newly discovered sequences can be compared to those available in the public databases and similar sequences identified. The benefits of comparison to sequences of known function are two-fold, not only does sequence similarity indicate the presence of a putative gene, the sequences also provide clues as to the putative function of a gene.

Submissions to the GenBank database (Benson *et al.*, 2004) have changed as methods of identifying single genes have been superseded by approaches aimed at identifying much larger numbers of genes with less focus on the genes' individual functions, including sequencing entire genomes. Genome sequencing began with the bacteriophage ØX174 (Sanger *et al.*, 1977) and sequencing projects now include the genomes of many fungi, for example *Magnaporthe grisea* (Dean *et al.*, 2005) and *Neurospora crassa* (Galagan *et al.*, 2003) genomes, animals, such as human (Venter *et al.*, 2001) and nematode (*Caenorhabditis elegans*; The *C. elegans* Sequencing Consortium, 1998) genomes, and plants, for example rice (*Orzya sativa*; International Rice Genome Sequencing Project, 2005) and *Arabidopsis thaliana* (The *Arabidopsis* Genome Initiative, 2000) genomes. Although sequence similarity searches have identified putative genes and gene structures within sequenced genomes, gene-finding programs developed to identify eukaryotic

gene structures in DNA sequence (Murakami and Takagi, 1998) have yielded a large number of uncharacterised putative genes with no sequence similarities. In addition to the large numbers of hypothetical genes now present in the publicly available databases, estimates suggest genome annotation has identified only 50-60% of the genes within most genomes (Sivashankari and Shanmughavel, 2006). However, the use of comparative genomics and expressed sequence tags (ESTs) has increased gene prediction accuracy (Stanke *et al.*, 2006).

The aim of this chapter was to identify any putative pathogenicity genes within the sequence flanking the integration site of the six non-pathogenic mutants using sequence similarity searches of the publicly available GenBank database.

6.2 Materials and Methods

6.2.1 Open Reading Frame Analysis

Open reading frames (ORFs) were identified using Vector NTI 10.3.0 (Invitrogen Australia Pty Ltd, Mount Waverley, VIC, Australia). Specifically, ORFs were identified and located if they were greater than 150 bp (50 amino acids) and originated from a putative ATG start codon and terminated at TAA, TGA or TAG stop codons. These settings represent the default given with ORF analysis by the Vector NTI 10.3.0 software (Invitrogen Australia Pty Ltd, Mount Waverly, VIC Australia). However, exon-intron structures cannot be identified using this program alone.

6.2.2 Sequence Similarity Searches

Database searches were performed using the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/) and the basic local alignment search tool (BLAST) (Altschul *et al.*, 1990). Both nucleic acid and amino acid sequences were submitted to the non-redundant database using the blastn, blastx and blastp functions (Altschul *et al.*, 1997) using the default settings, that compare queried nucleotide sequences to nucleotide sequences in the database, translate the queried nucleotide sequence in all three frames to amino acid sequence and compare to amino acid sequences in the database and compare queried amino acid sequences to amino acid sequences in the database, respectively.

Sequence alignments were performed using Vector NTI 10.3.0 software (Invitrogen Australia Pty Ltd, Australia).

6.3 Results

Sequences flanking the plasmid integration sites in six non-pathogenic mutants were analysed for the presence of candidate pathogenicity genes. Considering these were gene disruption mutants, special focus was given to the sequence at, and directly adjacent to the integration site. Each mutant was analysed individually and, consequently, results for each mutant will be presented separately.

6.3.1 Mutant YB4.20

In total, PCR walking generated 5072 bp of sequence for the mutant YB4.20 (Fig 6.1). Two-fold redundancy was achieved for 2838 bp of the total sequence (Fig 5.4).

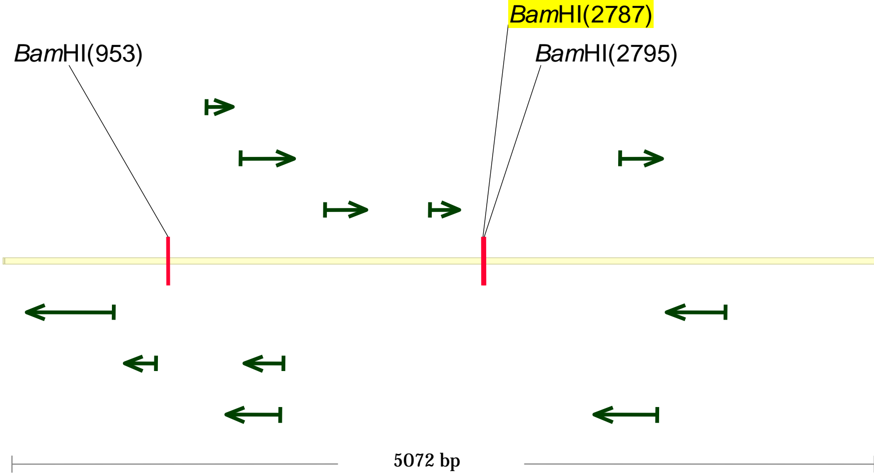


Figure 6.1: Location of putative ORFs (⟼) relative to the integration site present in all six possible reading frames. The *Bam*HI site of pAN7-1 integration is highlighted.

The available sequence was submitted to the public databases using both Blastn and Blastx. No significant sequence similarities were identified using either of these algorithms indicating that if any putative gene was present within the fungal sequence, no similar sequences were present in the database.

6.3.2 Mutant YB4.44

Blastx searches revealed sequence identity to database amino acid sequences corresponding to ORF 1, ORF 2 and ORF 3 (Fig 6.2). The start codon of ORF1 is located 140 bp downstream of the integration site. The strongest sequence identity was to a hypothetical *Aspergillus terreus* protein (3e-93; accession number EAU35705). However, sequence identity to a number of cytochrome P450 genes was also detected, the strongest to an alkane monooxygenase gene from the filamentous fungus *Graphium sp*. ATCC 58400 (1e-86; accession number AAR99474). The remaining ORFs did not produce any strong sequence similarities to the database.

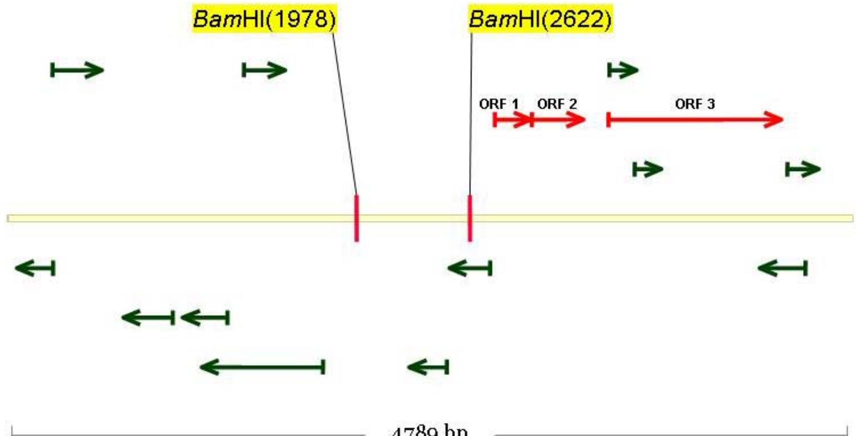


Figure 6.2: Location of ORFs (➔) with respect to the integration site located between the two highlighted *Bam*HI restriction enzyme sites. ORF's indicated in red have database similarity.

Two hypothetical proteins from *A. nidulans* FGSC A4 (accession numbers EAA58032 and EAA61133) that had gene information available were used to identify a potential intron. Using their nucleic acid and amino acid sequences their introns were identified and compared with the genomic sequence of YB4.44. Comparisons of the nucleic acid (see appendix DNA alignments) sequences were then used to identify the one putative intron (Fig 6.3).

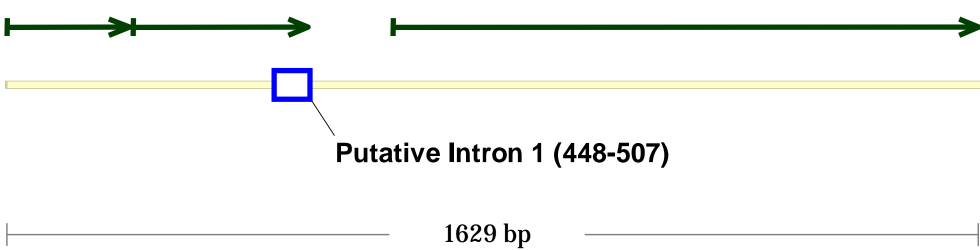
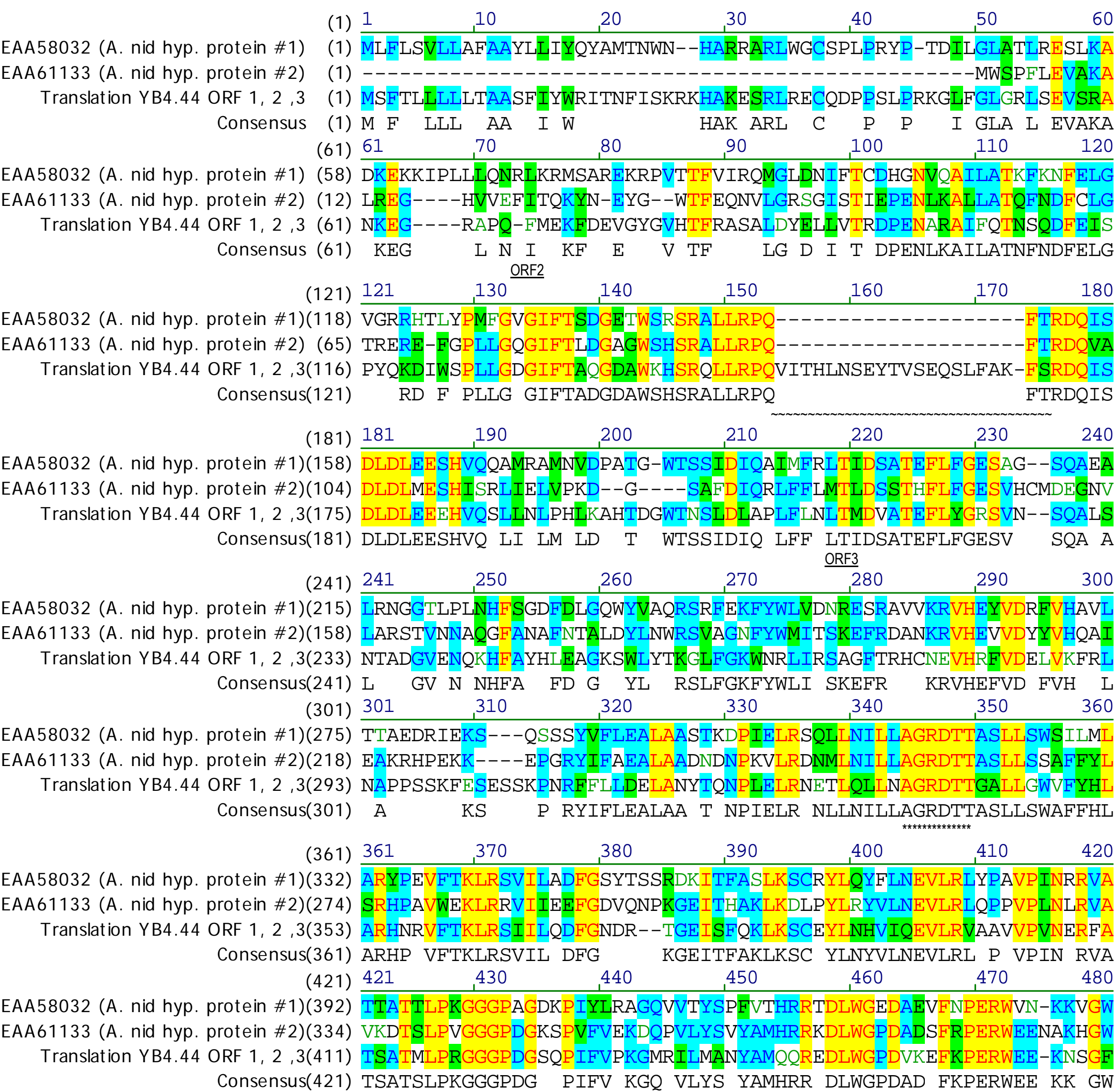


Figure 6.3: YB4.44's ORFs 1, 2 and 3 with one putative intron.



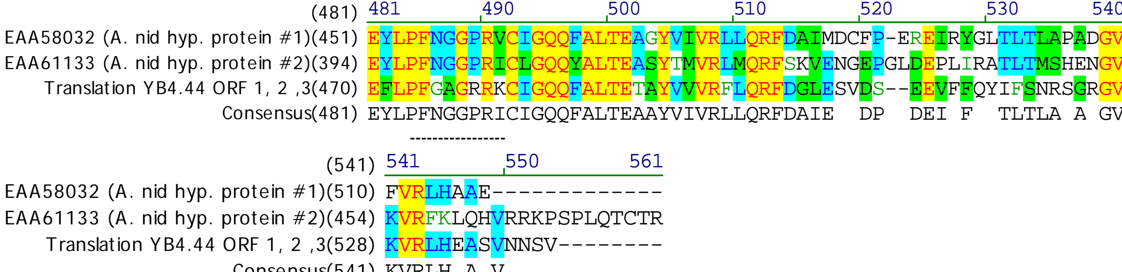


Figure 6.4: Amino acid alignment of the translation of ORF 1, 2 and 3 from mutant YB4.44 with two hypothetical proteins from *A. nidulans* (accession numbers EAA58032 and EAA61133). Alignment identity positions 23.7%; alignment consensus positions 69.3%. Underlined sequences: ~~~~~ putative intron, ┄┄┄ putative transmembrane helix and ------- putative heme binding region. <u>ORF2</u> and <u>ORF3</u>: the methionine residues (M) denoting the start of ORF2 and ORF3 in the translated YB4.44 amino acid sequence, respectively.

Upon removal of this intron the sequence became two ORFs encoding a total of 524 amino acids. This length is equivalent to, or slightly longer than (1-14 amino acids) other, similar, cytochrome P450 genes in the database. Although the possibility exists that a second intron is present at the end of ORF 1, subsequently removing the stop codon and combining the three ORFs into one single putative amino acid sequence, none of the other sequences had an intron at that location. If ORF 1 is not part of a larger putative gene structure, then a combination of ORF 2 and ORF 3 could form a putative gene. The start codon of ORF 2 begins 350 bp downstream of the integration site. Combining ORF 2 with ORF 3 and removing the putative intron results in an amino acid sequence 452 residues long. When submitted to the database, search results do not differ significantly from those returned by the three combined ORFs, ORF1 ORF2 and ORF3.

Both combinations of amino acid sequences were examined for the presence of two conserved domains, a transmembrane helix and a heme binding region (van den Brink *et al.*, 1998; Iida, 1998) that are present in cytochrome P450 amino acid sequences (Fig 6.4). The first, a transmembrane helix is conserved at 6 of 7 residues with a glycine to alanine substitution (AGRDTT<u>G</u>). The second conserved region, involved in heme binding, has four of the seven residues conserved, two synonymous and two non-synonymous amino acid substitutions (PF<u>GA</u>GRRK; highlighted=conserved and underlined=non-synonymous).

6.3.3 Mutant YB7.395

PCR walking produced a total of 3841 bp of sequence for mutant YB7.395. Sequence analysis that focused on and around the integration site showed there were no similar sequences present in the database. Furthermore, ORF analysis indicated that no significant ORF's were disrupted by the pAN7-1 integration (Fig 6.5).

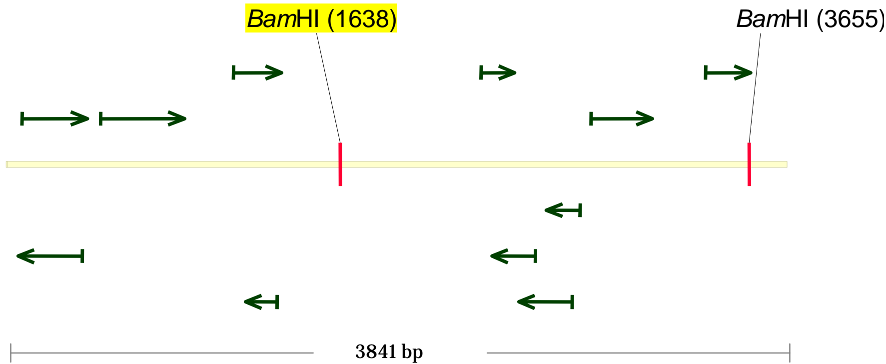


Figure 6.5: ORF ( ) and integration site (highlighted *Bam*HI site) location in mutant YB7.395.

6.3.4 Mutant YB7.412

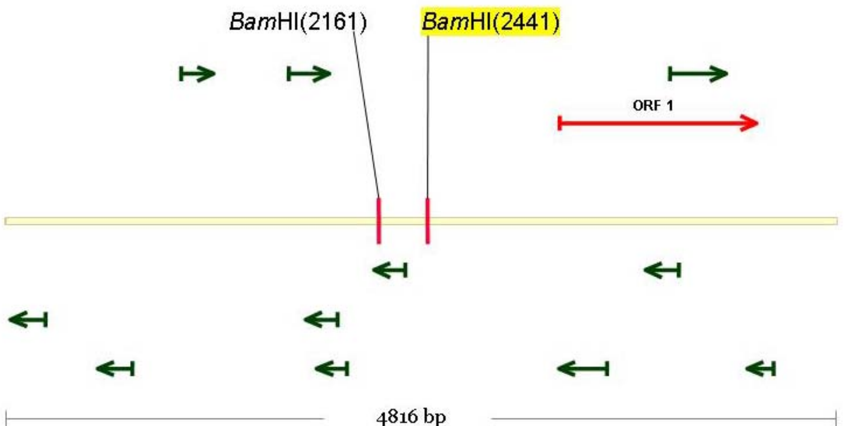


Figure 6.6: Location of ORFs ( ) with respect to the highlighted *Bam*HI integration site of mutant YB7.412. ORF's indicated in red have database similarity.

Blastx submission of the entire 4816 bp sequence of the integration site of mutant YB7.412 showed that one ORF had sequence identity to sequences in the public database. ORF 1 showed low sequence identity to hypothetical proteins from *Fusarium graminearum* (accession number: XP386314; 8e-48), *Chaetomium globsum* (accession number: EAQ90542; 4e-43) and *Neurospora crassa* (accession number: XP961159; 1e-40). ORF 1 is located 765 bp from the *Bam*HI site that integration occurred and encodes a 381 amino acid sequence (Fig 6.6).

6.3.5 Mutant YH4.5

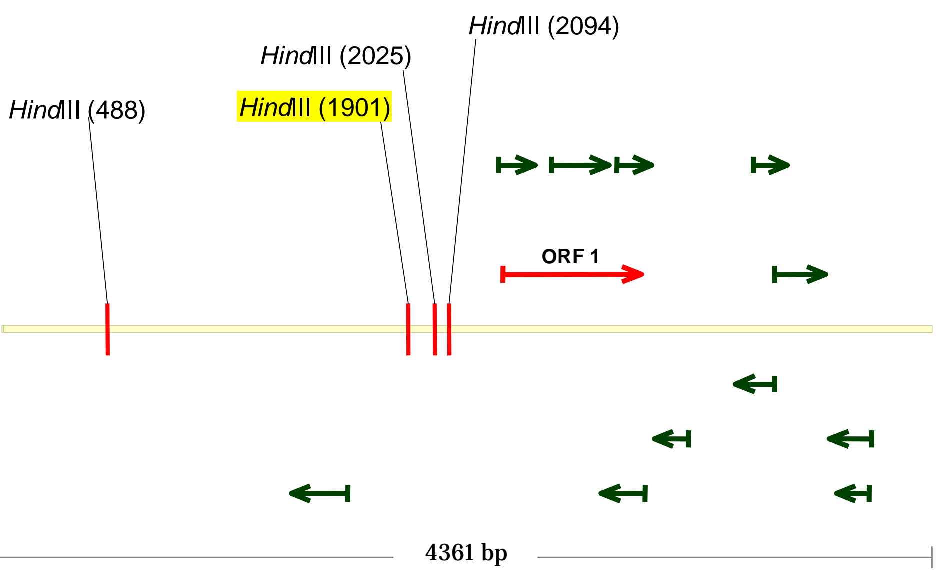


Figure 6.7: Location of integration site and ORFs (➡) of mutant YH4.5. The highlighted *Hind*III site is the location of pAN7-1 integration. ORF's indicated in red have database similarity.

Submission to the database revealed that the 217 amino acid sequence encoded by ORF 1 showed similarity to proteins of the rhomboid family (Fig 6.7). The strongest similarity to a rhomboid family protein (3e-12) was detected in *Tetrahymena thermophila* (accession number: XP001009676) (Eisen *et al.*, 2006). ORF 1 starts 443 bp from the *Hind*III integration site.

Upon translation of ORF 1, a common element to the rhomboid family proteins was observed. Several polar amino acids are conserved in nearly all members of the rhomboid family

(Koonin *et al.*, 2003); four out of five of the conserved amino acids were observed in ORF 1 of YH4.5.

6.3.6 Mutant LH2013

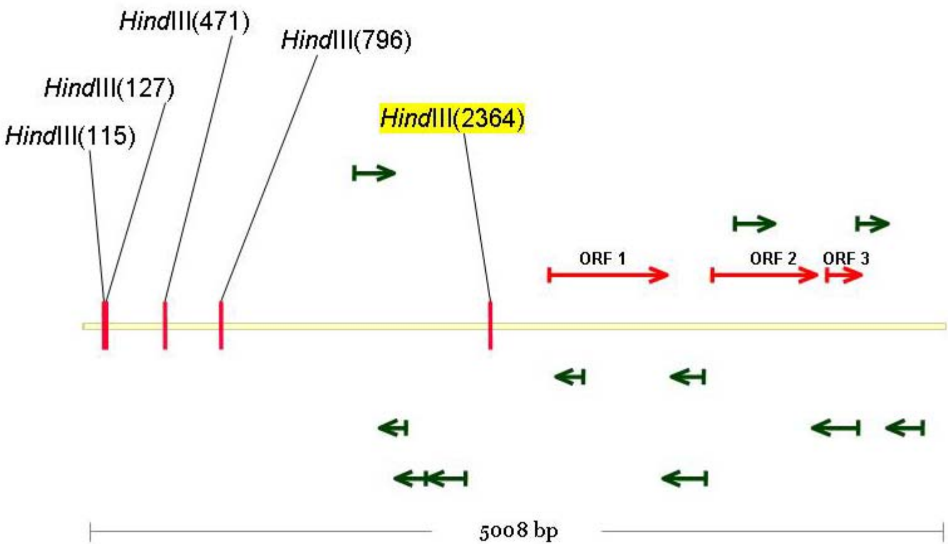


Figure 6.8: Location of ORFs (➞) with respect to the *Hind*III integration site (highlighted). ORF's indicated in red have database similarity.

Database searches of mutant LH2013 sequence indicated that sequence similarity was observed at ORF 1, ORF 2 and ORF 3. The start of ORF 1 is 340 bp from the *Hind*III integration site. Using similar hypothetical proteins and their respective gene sequences in the database, three putative introns were identified within the combined ORF 1, 2, and 3 sequences.

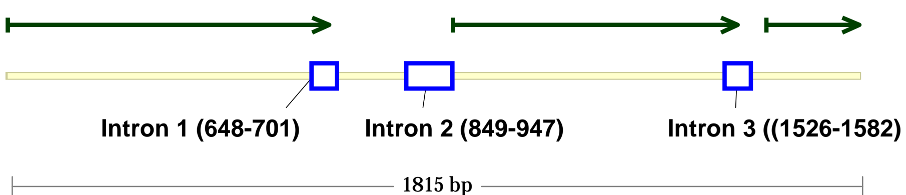


Figure 6.9: Location of putative introns within ORFs 1, 2 and 3.

Two hypothetical proteins from *Gibberella zeae* PH-1 (accession number: EAA78258) and *Magnaporthe grisea* (accession number: XM_363459), their mRNA sequences and genomic DNA sequences were used to identify the presence of potential introns within LH2013 ORFs 1, 2 and 3 (see appendix DNA alignments). Amino acid alignments of LH2013 with the two hypothetical proteins can be observed in Fig 6.10. Once introns were removed, the amino acid sequence is consistent with the proteins in the database in sequence length. Furthermore, the putative coding region is conserved to amino acid permease enzymes with a predicted 12 transmembrane domains (http://www.sbc.su.se/~miklos/DAS/).

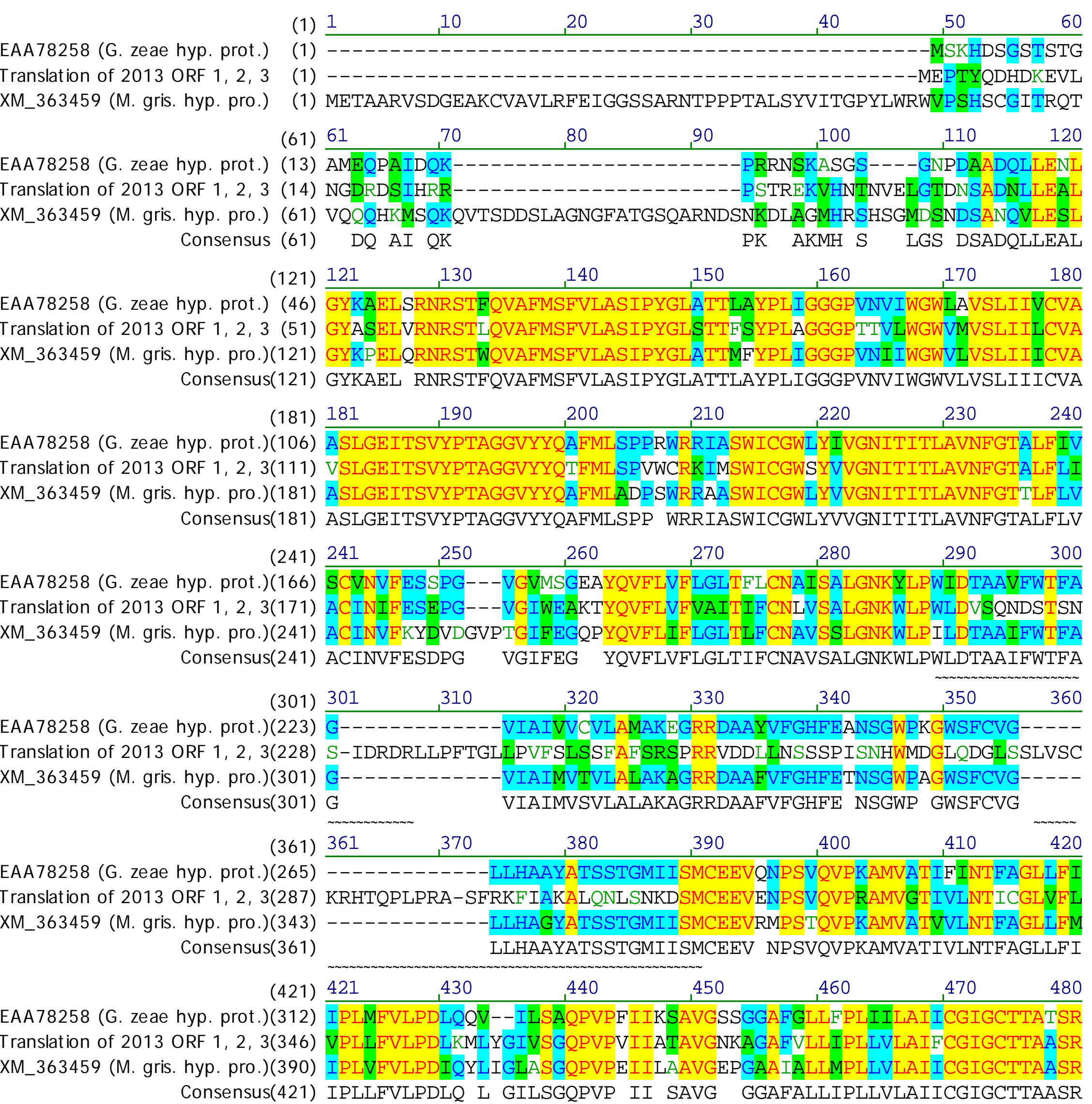


Figure 6.10: Amino acid alignment of the translation of ORF 1, 2 and 3 from mutant LH2013 with two hypothetical proteins from *G. zeae* (accession number: EAA78258) and *M. grisea* (accession number: XM_363459). Alignment identity positions 40.3%; alignment consensus positions 73.2%. Underlined sequence, ~~~~~ putative intron.

6.4 Discussion

6.4.1 Mutants Lacking Sequence Similarities

The lack of database sequence similarity at the integration site in mutants YB4.20 and YB7.395 suggests that a putative genes is not present, and therefore a loss of pathogenicity may not be the consequence of pAN7-1 integration. This is one of the greatest concerns when using REMI as a mutagenesis system because it suggests that the phenotype arose from genetic rearrangements elsewhere in the genome unlinked to the tag.

6.4.2 Location of Open Reading Frames with Respect to the Integration Site

Interestingly, none of the ORFs identified as putative genes were disrupted by the position of pAN7-1 integration. This finding is different compared to most other studies where plasmid

integration occurs within the ORF of the putative gene identified. However, this does not exclude the possibility that gene expression was decreased or silenced by integration within the genes' promoters and, consequently, gene function inhibited. The *pth3* gene in *M. grisea* was identified with a plasmid integration that was located in the gene's promoter. Integration reduced radial growth on minimal media to 40% the rate of the wild type. This mutant showed a partial requirement for histadine and contained a plasmid integration 240 bp upstream of translation initiation (Sweigard *et al.*, 1998). With respect to *R. secalis*, only a limited number of functional genes have been cloned. Furthermore, considering the diversity of eukaryotic promoters it is difficult to ascertain whether any active elements within the promoter sequence have been disrupted. The location of the integration sites with respect to the four mutants with ORFs of interest vary in distance to the putative start codon with the closest located only 140 bp from the integration (YB4.44) to the furthest 765 bp from the *Bam*HI integration site (YB7.412). Although no sequence information can determine the likelihood of promoter disruption for any of these putative genes, using simple logic; the closer that integration occurred to the start codon, the greater the probability that the putative gene's promoter has been disrupted.

6.4.3 Possible Gene Functions

Putative genes were identified near the integration site of four non-pathogenic mutants: YB4.44, YB7.412, YH4.5 and LH2013. Furthermore, three of these genes show similarities to genes or families of genes with known functions. The ORFs of interest in mutant YB4.44 show similarity to cytochrome P450 genes that are heme-thiolate enzymes that collectively catalyse a range of monooxygen reactions (Yadav and Loper, 1999; Yadav *et al.*, 2003; Deng *et al.*, 2007). Genes encoding cytochrome P450 enzymes have been reported to be involved in plant pathogenicity of fungi. One example of a plant pathogenicity gene is the pisatin demethylase (PDA) from *Nectria haematococca*, a pathogen to garden pea, *Pisum sativum* L.. PDA demethylates the antimicrobial phytoalexin pisatin (VanEtten *et al.*, 1989). Another cytochrome P450 gene involved in plant pathogenicity was isolated from the plant pathogenic fungus *Fusarium sporotrichioides*. Virulence enhancement in *F. sporotrichioides* relies on the production of sesquiterpenoid trichothecene and the *Tri4* gene is involved in the trichothecene biosynthesis pathway (Hohn *et al.*, 1995).

Amino acid permease similarity was detected from database searches of LH2013 ORFs. Essentially these permeases act as sensing systems for extra cellular stimuli to access nutrients from the surrounding environment. Nutrient sensing is important in general fungal development, and more specifically in pathogenicity (Smith *et al.*, 2003). The Gap1 permease from *S. cerevisiae* acts as a sensor for protein kinase A after nitrogen is supplied to yeast cells starved of that nutrient (Donaton *et al.*, 2003). In *A. nidulans* the *gabA* gene encodes a permease that allows the uptake of the carbon, nitrogen source γ–amino-n-butyrate (Hutchings *et al.*, 1999). Nutrient uptake is crucial in the early stages of host colonisation.

The sequence flanking the plasmid insertion in mutant YH4.5 showed similarities to sequences encoding proteins of the rhomboid family of intramembrane proteases. Rhomboid (Rho) proteases are a conserved group of integral membrane proteins that cleave proteins in a multitude of prokaryotic and eukaryotic signalling pathways (Urban and Freeman, 2002; van der Bliek and Koehler, 2003). Rhomboid proteases were initially characterised in *Drosophila melanogaster,* where they initiate cell signalling during development (Urban and Freeman, 2003). Cell-cell signalling is important in a number of cellular processes including responses to developmental and environmental changes (Urban, *et al.*, 2001). For example, in *M. grisea* a mitogen-activated protein (MAP) kinase, *PMK1*, was shown to be essential for appresorium formation and pathogenesis. Rhomboid proteases have also been implicated in fungal pathogenicity. *Fusarium oxysporum* mutants lacking *rho1* showed decreased virulence on tomato plants (Martinez-Rocha *et al.*, 2008).

Chapter 7 – Functional Analysis

7.1 Introduction

The final step in this study was to seek definitive proof that the four putative genes discussed in chapter 6 were inactivated by plasmid insertion, and that gene inactivation was responsible for the mutant's loss of pathogenicity. Due to a lack of time and resources two genes were selected for further analysis, the putative permease gene and the putative cytochrome P450 gene. The respective mutants LH2013 and YB4.44 were selected because they showed stronger sequence similarity to known functional genes than the other mutant's putative genes (see 6.3.1-6.3.6), and their plasmid integration sites were closer to the start codons of the ORFs. Furthermore, these two genes were selected because they represented the two different fungal strains, strain 5 and UK7.

To prove a gene's involvement in pathogenicity two contrasting methods can be used, complementation and knock out studies. Complementation, a gain-of-function approach, determines if returning the fully functional wild type gene into the non-pathogenic REMI mutant can return virulence. Alternately, knock out studies, a loss-of-function approach, involves the targeted disruption of the putative pathogenicity gene in the wild type fungus using homologous recombination (HR) that reproduces the non-pathogenic phenotype. While both methods have their positive and negative attributes, the functional analysis of these genes was carried out using knock out studies. Before outlining the reasoning behind this decision, an overview of the success and limitations of each method is given.

Returning the fully functional wild type gene into the non-pathogenic REMI mutant can return virulence to non-pathogenic mutants. Complementation has been used as a tool to study pathogenicity genes in *Colletotrichum graminicola* (Thon *et al.*, 2000) and *Fusarium oxysporum* (Namiki *et al.*, 2001). However, the fact that functional complementation required the transfer of the wild type gene to the non-pathogenic mutant made it a less desirable than the alternative methodology. Using the non-pathogenic mutants in transformation studies posed two significant problems. Firstly, at this point in the study the PEG/$CaCl_2$ transformation protocol was, in other experiments, producing transformants at such low frequencies that it was no longer a viable

transformation system. Consequently, the alternative was the ATMT system that transfers DNA from *A. tumefacincs* cells to fungal spores. Obviously, with the non-sporulating stain 5 mutants this methodology was not possible. The development of an alternate ATMT system that utilised mycelia as a substitute for fungal spores was considered. Hyphal fragments have been successfully transformed by ATMT in other fungi, for example *Venturia inaequalis* (Fitzgerald *et al.,* 2003), *Acremonium implicatum* (Abello *et al.*, 2008), *Rhizoctonia solani* (Wu, 2003), *Cadophora finlandia* and *Phialocephala fortinii* (Gorfer *et al.,* 2007). The development of this transformation system was not possible due to time constraints. Secondly, the inability of strain 5 to produce spores is representative of a loss of general viability in the fungal mutant strains. Morphologically, the selected UK7 and strain 5 mutants were showing reduced fitness illustrated by slower growth rates and reduced fungal biomass produced in culture (data not shown). *R. secalis* can not be continually transferred and maintained *in vitro* without negative effects on fitness. Considering the number of transfers involved in transformation and subsequent multiplication before inoculation, there was concern that mutants would be seriously restricted in their growth *in vitro* prior to inoculation, if a complementation transformation were attempted. To summarise, complementation transformation on the strain 5 mutant was, given the available methodology, impossible, and the generations involved in regenerating the fungus following the UK7 mutant's transformation was considered likely to result in a loss of the mutant's viability. Therefore, knock out studies were considered the superior alternative for functional analysis of the selected, putative genes.

Although HR *in vivo* is a poorly understood process, studies have been performed in a variety of organisms using different strategies. Gene targeting is routine in the embryonic stem cells of mice where thousands of loss-of-function mutations have been generated. Furthermore, the numerous experiments performed indicate that approximately one homologous recombination event can be expected per one hundred random integration events in embryonic stem cells (Jasin *et al.*, 1996). This is considerably different in plant systems where the frequencies of homologous recombination events are often very low (Puchta, 2002). *A. tumefaciens*-mediated transformation of *Saccharomyces cerevisiae* revealed that T-DNA with homology to the yeast genome preferably integrates by homologous recombination (Bundock *et al.*, 1995; van Attikum and Hooykaas, 2003). Alternatively, where the T-DNA is non-homologous, integration in yeast occurs randomly by illegitimate recombination (Bundock and Hooykaas, 1996) consistent with the integration observed in plants (Pucta, 2002). *A. tumefaciens*-mediated HR has been successfully used in filamentous

fungi, including *Aspergillus awamori* (Gouka *et al.*, 1999) and the fungal phytopathogens *Colletotrichum gloeosporioides* (Stephenson *et al.*, 2000) and *Mycosphaerella graminicola* (Zwiers and De Waard, 2001), at variable but generally low frequencies.

To overcome the expected low frequency of HR, a rapid high throughput assay to identify potential knock out mutants of the two selected genes was devised involving a rapid DNA extraction, reducing the need for fungal biomass and culture time, and PCR based screening. PCR-based screening has successfully identified gene disruption mutants in other organisms including *Magnaporthe grisea* following the targeted disruption of the hydrophobin gene *MHP1* (Kim *et al.*, 2005).

7.2 Materials and Methods

7.2.1 Fungal Strains and Mutants

The fungal strains UK7 and 5 have previously been discussed (see 2.2.1). A sporulating strain 5 sample was selected that had undergone the least number of generations within the laboratory's collection. Furthermore, this sample was expected to produce adequate spores for inoculation after the generations required to generate a knock out mutant using ATMT as the transformation protocol.

7.2.2 HR Vector Construction

Two regions of DNA sequence, identified from mutants YB4.44 and LH2013, were selected for KO studies and, therefore, two HR vectors were constructed. Both vectors were based on the binary vector backbone pPZP200 (Hajdukiewicz *et al.*, 1994). The construction of both vectors is described in the following.

<u>YB4.44 KO vector</u>: Initially, primers 4.44Sbffwd and 4.44Xmarev amplified a 4785 bp fragment under long range PCR cycle conditions (see 5.2.3.4) from strain 5 wild type DNA as template. Both primers were designed to contain the *Sbf*I and *Xma*I restriction sites, respectively, to facilitate later cloning steps. The amplified fragment was separated from unused PCR reactants on a 1% agarose/TAE gel (1% w/v agarose dissolved in TAE buffer: 40 mM Tris-acetate, 1 mM EDTA, pH

8.0), stained with ethidium bromide, excised from the gel with a sterile scalpel blade under ultraviolet light, purified using the QIAquick® Gel Extraction Kit (Qiagen Pty Ltd, Doncaster, VIC, Australia) and then ligated into the pGEM®-T Easy Vector System II (Promega, Sydney, NSW, Australia), following manufacturer's instructions to create the 4.44 KO – 1 vector (Fig 7.1). Ligated plasmids were then transferred to competent *E. coli* cells, strain DH5α (see 2.2.5.2 – 2.2.5.3) and plated onto selective LB media. A single bacterial colony that contained the 4785 bp fragment was selected after PCR screening with primers 4.44check#1 and 4.44check#2. A liquid culture, LB broth and the antibiotic ampicillin (100 µg/ml), of the corresponding bacterial colony was grown for 16 hours at 300 rpm and 37°C and, following incubation, a small-scale plasmid DNA extraction and purification was performed on the liquid culture using the Qiaprep® Spin Miniprep Kit (Qiagen Pty Ltd, Doncaster, VIC, Australia), following manufacturer's instructions. The resulting vector DNA was then digested with *Bam*HI (New England Biolabs, Ipswich, MA, USA) and subsequently treated with calf intestinal alkaline phosphatase (Invitrogen Australia Pty Ltd, Mount Waverley, VIC, Australia), according to the manufacturer's instructions. Following de-phosphorylation, the cleavage products, 644 bp and 7157 bp, were separated on a 1% agarose/TAE gel, stained with ethidium bromide and visualised under UV light, and the 7157 bp fragment excised from the gel and purified with QIAquick® Gel Extraction Kit (Qiagen Pty Ltd, Australia).

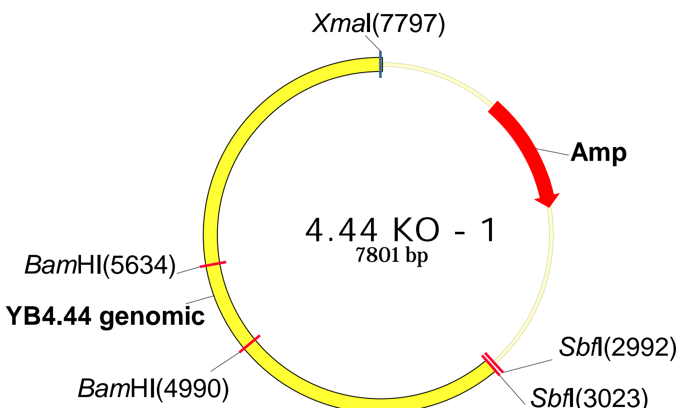


Figure 7.1: The 4.44 KO – 1 vector, fungal DNA from the YB4.44 integration site ligated into the pGEM®-T Easy Vector. Amp – ampicillin resistance gene.

A 3338 bp fragment of pAN7-1 was used as the disruptive element in the YB4.44 KO vector. This fragment contained the *gpdA* promoter and the hygromycin resistance gene coding

region but not the trpC terminator sequence from pAN7-1 (Punt *et al.*, 1987). Initially, a 3418 bp fragment, amplified with primers hphBamHIfwd and hphBamHIrev from pAN7-1 vector as template, was engineered to have *Bam*HI restriction sites at both ends of the fragment. The amplification product was separated from unused PCR reactants and excised from a gel, purified, ligated into the pGEM®-T Easy Vector and transferred to competent *E. coli* DH5α cells (see 2.2.5.2 – 2.2.5.3). Following a small scale DNA extraction using the Qiaprep® Spin Miniprep Kit (Qiagen Pty Ltd, Australia), DNA sequencing with the T7 and SP6 universal primers confirmed the presence of the amplification product. The selected vector was digested with *Bam*HI, its cleavage products, 3338 bp and 3095 bp, separated on a 1% agarose/TAE gel, stained with ethidium bromide, visualised under UV light and the 3338 bp band excised and purified, as previously described.

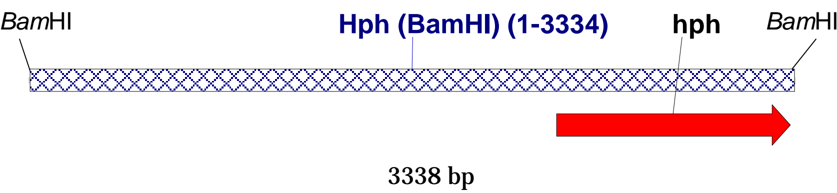


Figure 7.2: DNA amplified from pAN7-1 DNA as the template with primers hphBamHIfwd and hphBamHIrev and digested with *Bam*HI. hph – hygromycin resistance gene.

To maintain consistency with the YB4.44 non-pathogenic REMI mutant, the disruptive element was inserted into the *Bam*HI sites at which pAN7-1 had integrated into the fungal genome (Fig 5.5, Fig 5.12, Fig 6.2). To accomplish this, the *Bam*HI digest products containing YB4.44 genomic DNA and the hygromycin resistance gene (*hph*) were ligated using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) (Fig 7.3). To ensure read throughs were not possible the *hph* gene was inserted in the opposite direction to the ORFs with homology to cytochrome p450 genes (section 6.3.2). Orientation of the *hph* gene was determined by *Nco*I (New England Biolabs, Ipswich, MA, USA) cleavage that yielded two fragments, 5661 bp and 4834 bp (data not shown).

*Sbf*I and *Xma*I (New England Biolabs, USA) digests were performed on the 4.44 KO - 2 vector and the pPZP200 binary vector to release fragments of 7468 bp and 6508 bp, respectively, which were excised from a gel of the separated digestion products. Once purified with a QIAquick® Gel Extraction Kit (Qiagen Pty Ltd, Doncaster, VIC, Australia) the products were ligated with the T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) to produce the YB4.44 KO vector (Fig 7.4).

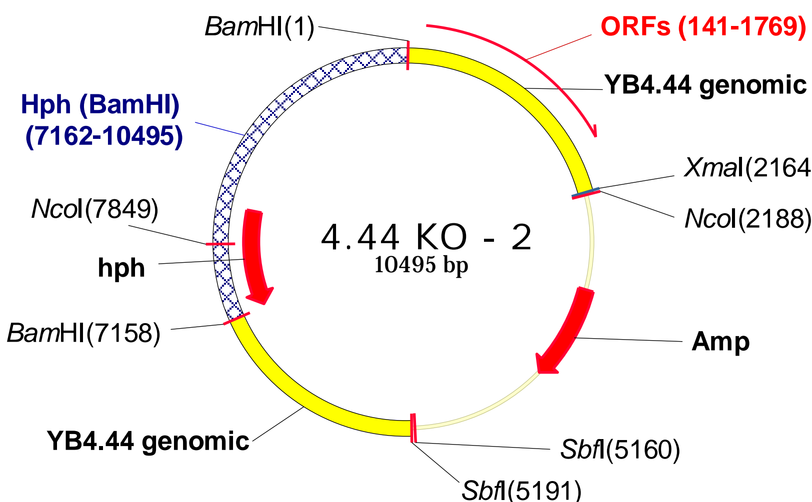


Figure 7.3: The 4.44 KO - 2 vector. A 3338 bp fragment amplified from pAN7-1 ligated into the original integration site of mutant YB4.44. Amp – ampicillin resistance gene, hph – hygromycin resistance gene, ORFs – open reading frames are indicated with thin red arrows.

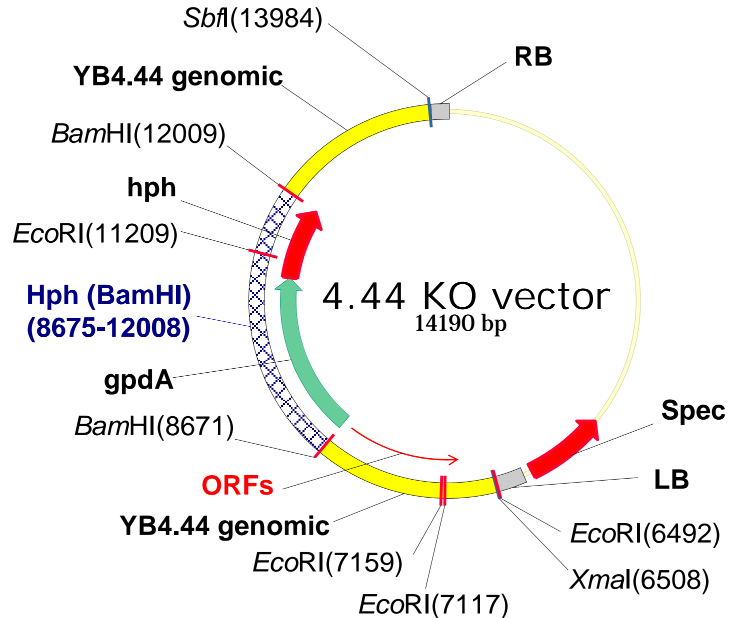


Figure 7.4: The YB4.44 KO vector. RB – right border, LB – left border, gpdA – *gpdA* promoter, hph – hygromycin resistance gene, Spec – spectinomycin resistance gene, ORFs – open reading frames are indicated with thin red arrows.

The vector was transferred into competent *E. coli* cells (see 2.2.5.2 – 2.2.5.3) and several transformant colonies grown in liquid culture. This process was identical to that previously described above with the exception that spectinomycin was used for antibiotic selection. To ensure the ligation reaction was successful, bacterial colonies were grown in liquid culture and plasmid DNA extracted and cleaved with *Eco*RI (New England Biolabs, USA) to yield four fragments, 9473 bp, 4050 bp, 625 bp and 42 bp (data not shown).

<u>LH2013 KO vector</u>: Initially a 5008 bp fragment was amplified from UK7 wild type DNA as the template using the 2013Bamfwd and 2013Sacrev primers. Both primers were specifically designed to contain the *Bam*HI and *Sac*I restriction sites, respectively, to facilitate later cloning steps. This PCR fragment was separated from unused PCR reactants on a 1% agarose/TAE gel, excised, purified, ligated into the pGEM®-T Easy Vector System II and transferred into *E. coli* DH5α cells, as previously described for the construction of the 4.44 KO vector. To ensure fragment ligation, several transformant bacterial colonies were grown in liquid culture and their extracted plasmids digested with *Eco*RI. One successful ligation reaction produced the expected bands of 5028 bp and 2997 bp (data not shown). Sequential *Bam*HI and *Sac*I (New England Biolabs, USA) digests were performed on the selected plasmid DNA, containing the fungal DNA from the integration site of LH2013, and also on the pPZP200 binary vector that released the desired 4998 bp and 6730 bp fragments, respectively, from the two vectors. Both fragments were excised from 1% agarose/TAE gels and purified by the previously described methods. The purified fragments were then ligated using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) and transferred to competent *E. coli* DH5α cells. Several bacterial colonies were selected and grown in liquid culture, using spectinomycin for antibiotic selection, and plasmid DNA extracted from the bacterial culture using the Qiaprep® Spin Miniprep Kit (Qiagen Pty Ltd, Australia). The resulting vector, named 2013 KO - 1 (Fig 7.5), was then cleaved with *Nco*I to ensure the expected ligation product was produced. *Nco*I cleavage gave the expected bands 10249 bp, 1130 bp, and 349 bp.

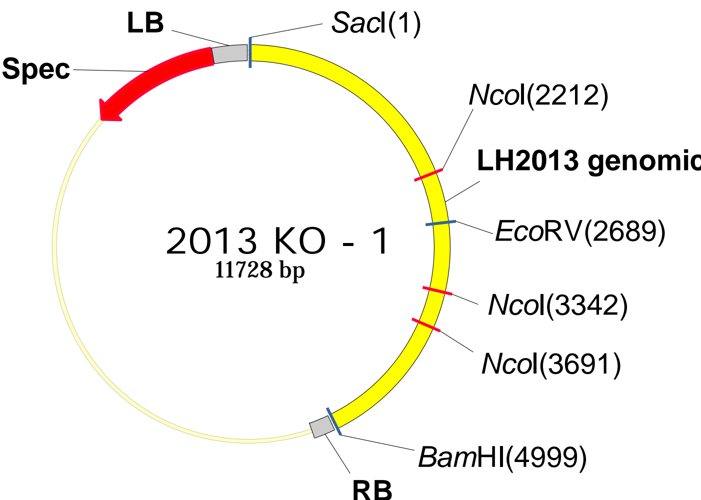


Figure 7.5: The 2013 KO – 1 vector. A 4998 bp fragment of fungal DNA from the LH2013 integration site ligated into the pPZP200 binary vector using *Sac*I and *Bam*HI enzymes. RB – right border, LB – left border, Spec – spectinomycin resistance gene.

It was not possible to locate the disruptive element in the 2013 KO vector as pAN7-1 was positioned in the LH2013 REMI mutant because multiple *Hind*III sites were observed in the sequence flanking the integration site of LH2013 (Fig 5.9, 5.13, Fig 6.8). Consequently, an *Eco*RV site was used to insert a 3606 bp fragment, containing the *gpdA* promoter driving *hph*, 10 bp upstream of LH2013's ORFs showing homology to permease genes (section 6.3.6). This was similar to the original plasmid integration position within the putative gene's promoter. Initially, this fragment was amplified with the hphEcoRVfwd and hphEcoRVrev primers engineered to contain *Eco*RV restriction sites. The 3612 bp fragment amplified was separated on a 1% agarose/TAE gel, excised, purified, ligated into the pGEM®-T Easy Vector, transferred to *E. coli* and the plasmid multiplied in bacterial liquid culture. Following plasmid extraction, the T7 and SP6 universal primers were used in a PCR to confirm that the integrated fragment was of the correct length and the plasmid's identity was proven by sequencing with T7 and SP6. *Eco*RV cleavage released the 3006 bp fragment that was separated by electrophoresis, excised and purified by the previously described methods (Fig 7.6).

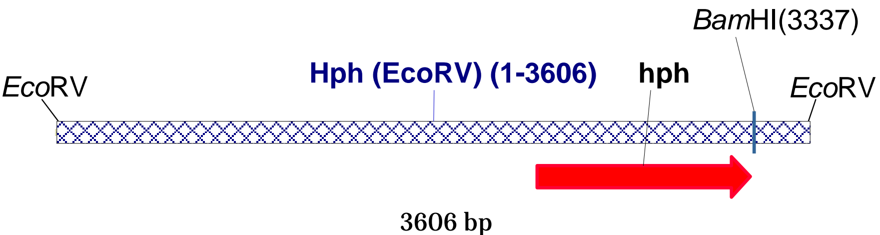


Figure 7.6: pAN7-1 fragment deliberately engineered to contain the hygromycin resistance gene and the *gpdA* promoter with terminal *Eco*RV ends.

The 2013 KO – 1 vector was then cleaved with *Eco*RV and dephosphorylated with calf intestinal alkaline phosphatase (Invitrogen Australia Pty Ltd, Mount Waverley, VIC, Australia), according to the manufacturer's instructions and ligated to the disruptive element (Fig 7.7). The resulting vector was transferred to competent *E. coli* DH5α cells and several colonies were selected for liquid culture, with spectinomycin antibiotic selection, and plasmid DNA extraction. To ensure successful ligation and that orientation of the disruptive element was in the opposite direction of the ORFs of interest a *Bam*HI (New England Biolabs, USA) digest was performed. Following *Bam*HI cleavage, the expected bands of 9684 bp and 5650 bp were observed (data not shown). Complementary orientation of the disruptive element was necessary to prevent any potential read throughs from the *gpdA* promoter to the ORFs of interest.

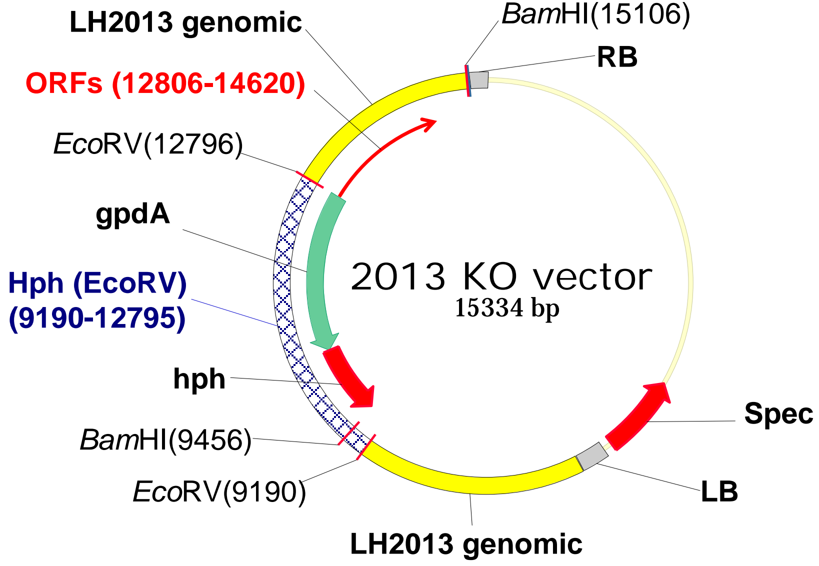


Figure 7.7: The LH2013 KO vector. RB – right border, LB – left border, gpdA – *gpdA* promoter, hph – hygromycin resistance gene, Spec – spectinomycin resistance gene.

7.2.3 Materials and Methods for Handling *Agrobacterium tumefaciens*

The materials and methods for *A. tumefaciens* handling, transformation and use in fungal transformation are detailed in Chapter 2.

7.2.4 DNA Isolation from Fungal Colonies for PCR

Half of a 5 mm diameter fungal colony was added to 300 µl of extraction buffer (200 mM Tris/HCl, 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS, pH 8.5) and ground with the point of a knitting needle until a homogenate was produced. To the homogenate 150 µl of 3 M sodium acetate (pH 5.2) was added, the tube incubated at -20ºC for 10 min and then centrifuged at 13000 rpm for 20 min. The supernatant was removed, mixed with an equal volume of isopropanol and incubated at room temperature for 5 min. The mixture was spun for 20 min at 13000 rpm to produce a pellet. This pellet was washed with 70% (v/v) ethanol, dried and resuspended in 50 µl of nanopure H_2O.

7.2.5 PCR Identification of Homologous Recombination Events

Three primers were used in each amplification reaction to identify homologous recombination events. Two ‘genomic’ primers were designed to anneal to genomic DNA directly flanking the disruptive element of the YB4.44 KO vector and the LH2013 KO vector. A third ‘knock out’ primer was designed to anneal to the disruptive element of the two respective vectors, facing the genomic DNA adjacent to the disruptive element. Hence, amplification reactions could potentially produce two bands: a smaller wild type band amplified by the genomic primers, and a larger knock out band amplified by one of the genomic primers and the knock out primer. A smaller band was indicative of an intact genomic region and, therefore, failure of a homologous recombination event occurring. The presence of both bands represented intact genomic DNA and an ectopic T-DNA integration. However, the larger band was often not visible because PCR preferentially amplifies smaller fragments. If homologous recombination had occurred, only the larger band would have been visible.

Two three PCR primer sets were initially used to identify potential knock out mutants from mutants transformed with the T-DNA regions of the YB4.44 KO vector and the LH2013 KO vector.

Later, new primers sets were designed to increase the efficiency of PCR results and screening reactions. The primer combinations and their respective expected band sizes are given in Table 7.1.

7.3 Results

7.3.1 Transformation Results

Three separate ATMTs of both parental strains with the appropriate KO vector were performed. This number of transformations provided in excess of 200 transformants for each KO vector. In these transformations, efficiency was never calculated because the success of these experiments was judged on the production of enough mutants to a screen for a KO event.

7.3.2 PCR Results

Initially, 90 fungal colonies were screened with the primer combination 4.44check#1, 4.44seq#3 and 4.44hph (Fig 7.8(A)). Of these 90 colonies, only two produced visible bands. In both cases a wild type band was observed indicating that a homologous recombination event had not occurred. To improve the efficiency of the PCR screening process a new primer combination was employed. These primers had significantly smaller amplification products and were considerably more efficient. With the primers 4.44KO1, 4.44 KO2 and 4.44hph (Fig 7.8(C)) 200 transformant colonies were screened and in all cases except one (data not shown), a wild type band was observed. Two repeat PCR reactions were performed on the single sample that had failed to produce a wild type band in the first PCR. In both reactions a wild type band was amplified demonstrating HR had not occurred.

Transformant colonies of the fungal strain UK7, produced with the LH2013 KO vector, were screened with the primer combination 2013 1, 2013KOcheck and 2013hph (Fig 7.8(B)). In total 480 separate reactions were performed yielding 229 products that contained the wild type band. To improve efficiency the primer combination 2013KO1, 2013KO2 and 2013hph (Fig 7.8(D)) were used and successfully screened 116 further mutant colonies. In all 345 cases the wild type band was amplified showing that no HR event occurred.

Table 7.1: Primers used to screen homologous recombination events in strain 5 and UK7 mutants transformed with YB4.44 KO vector and LH2013 KO vector. All band sizes are shown in base pairs.

Transformed with YB4.44 KO Vector			Transformed with LH2013 KO Vector		
Primer Combination	Band Size	Band Type	Primer Combination	Band Size	Band Type
4.44check#1 + 4.44seq#3	1087	WT	2013 1 + 2013KOcheck	740	WT
4.44check#1 + 4.44hph	1240	KO	2013 1 + 2013hph	1010	KO
4.44KO1 + 4.44KO2	303	WT	2013KO1 + 2013KO2	324	WT
4.44KO1 + 4.44hph	527	KO	2013KO1 + 2013hph	626	KO

WT – Wild type band

KO – Knock out band

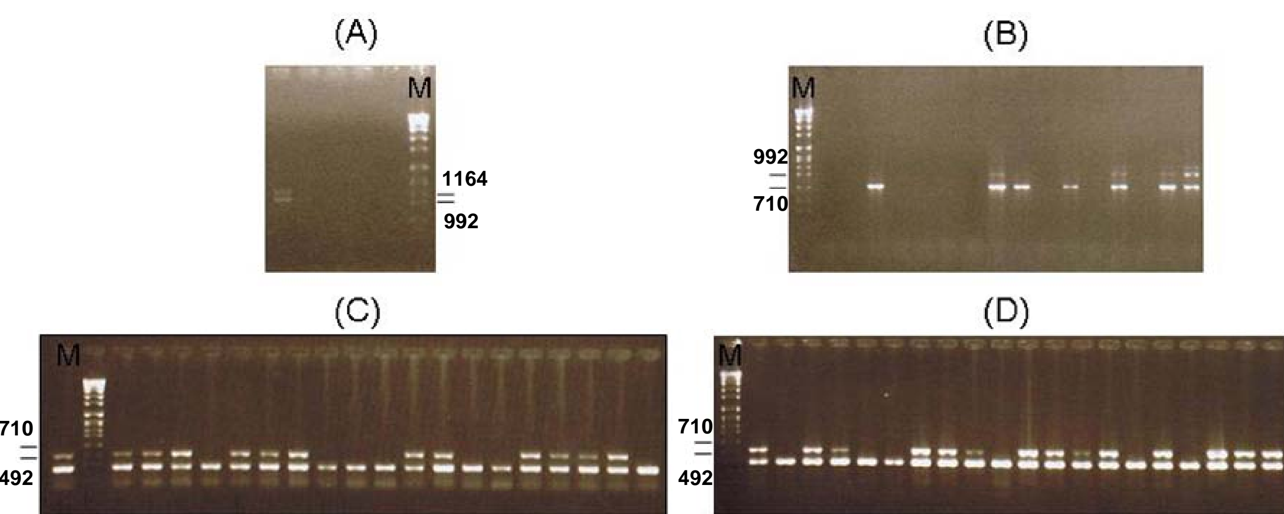


Figure 7.8: Ethidium bromide stained PCR products separated on 1% agarose/TAE gels of the following reactions: (A) Strain 5 DNA transformed with the YB4.44 KO vector, amplified with primers 4.44check#1, 4.44seq#3 and 4.44hph; (B) strain UK7 DNA transformed with LH2013 KO vector, amplified with primers 2013 1, 2013KOcheck and 2013hph (C) strain 5 DNA transformed with the YB4.44 KO vector amplified with primers 4.44KO1, 4.44KO2 and 4.44hph; (D) strain UK7 DNA transformed with LH2013 KO vector, amplified with primers 2013KO1, 2013KO2 and 2013hph. Each lane in the gels represents a separate mutant and PCR reaction with the exception of those lanes marked with an M that represent SPP-1 Phage DNA digested with the *Eco*RI restriction enzyme (Geneworks Pty Ltd., Hindmarsh, SA, Australia). Indicators for marker band sizes are represented in base pairs and band sizes for SPP-1 Phage DNA digested with *Eco*RI are presented in the appendices. 'Empty' lanes represent failed amplification reactions.

7.4 Discussion

7.4.1 Failure of HR Occurring

Two possible reasons were identified to account for the failure to generate a fungal knock out mutant, the length of flanking sequence used in the knock out vector, and the number of mutants tested for an HR event. Limited HR and targeted gene disruption research has been undertaken in *R. secalis* and this HR study would have greatly benefited from a preceding study that detailed HR in this fungus. With limited prior research, it was difficult to decide with certainty on the amount of flanking sequence that would optimise the occurrence of a HR event and experimental investigation comparing flanking sequence length and HR efficiency could not be performed due to time constraints. Reported HR studies in other filamentous fungi demonstrate that the amount of flanking sequence used in the knock out vectors varies significantly. The targeted disruption of the *MHP1* gene in *Magnaporthe grisea* utilised 2.5 kb of homologous sequence on both flanks of the knock out vector to facilitate a HR event (Kim *et al.*, 2005). In *Mycospaerella graminicola* efficient disruption, approximately 44% disruptants, was achieved using ATMT and 2.6 kb and 4.1 kb of flanking homologous DNA (Zwiers and De Waard, 2001). In contrast, a high frequency of HR occurred in *Colletotrichum gloeosporioides* in the disruption of the *CgDN3* gene using homologous sequences 744 and 335 bp in length and Colot *et al.* (2006) states wild type *N. crassa* strains have low frequencies of HR, less than 10%, even when using knock out vectors containing large amounts of flanking sequence. The only previously successful disruption studies in *R. secalis* were those performed on three necrosis inducing peptide genes, *nip1*, *nip2* and *nip3*. Disruption in each case used varying lengths of flanking sequence in the knock out vectors. Genes *nip1* and *nip3* were disrupted using the PEG/$CaCl_2$ method of transformation with vectors containing 1116 bp and 1549 bp of flanking sequence, and 1226 bp and 1334 bp of flanking sequence, respectively. Comparatively, the *nip2* gene was disrupted using ATMT with 910 bp and 1412 bp of flanking sequence in the disruptive element (W. Knogge, pers. comm.). For HR studies on the mutants YB4.44 and LH2013, larger amounts of flanking sequence, 2163 bp and 1975 bp, and 2688 bp and 2310 bp, respectively, were employed to encourage homologous sequence association and recombination. Although these sequences were larger than those used to disrupt the *nip* genes, in

this case there were larger amounts of flanking sequence surrounding the target of interest and therefore more sequence available to use in a HR procedure. Consequently, following the logic that the greater the length of flanking sequence the greater the chance of homologous DNA association, the complete sequence available was used in the HR study.

Time and resources have always been the two greatest limiting factors that restrict scientific studies. In this study, these factors prevented the generation of a successful targeted gene disruption mutant. Time and resource availability would have allowed a more detailed HR study to be performed, and increased the chance of producing a successful knock out mutant. Both the number of mutants tested and sequenced, and the loss of sporulation that strain 5 exhibited during the course of the study were factors that severely reduced the time available to perform HR studies.

In total, 10 non-pathogenic mutants were identified through phenotypic screening. After Southern analysis, six mutants were selected for further analysis. In hindsight, this number was excessive considering the laborious and time consuming nature of PCR walking and sequencing. Six mutants would have been a more appropriate number if targeted gene disruption was a more routine process in *R. secalis*. Furthermore, if only one rather than two non-pathogenic mutants were selected for knock out studies, a second knock out vector with less flanking sequence could have been constructed for that mutant to determine if less flanking sequence improved HR efficiency.

No scientific endeavour can be considered a linear passage from hypothesis to conclusion and some deviation from the dogmatic approach established at this study's inception was expected. However, the loss of sporulation in fungal strain 5 after five generations could not have been anticipated and was a major and time consuming distraction. Strain 5 was initially selected in the project to overcome quarantine restrictions applied to the imported European strain UK7. This indigenous strain was also considered to be more virulent than other Australian strains (L. Scott, pers. comm.). During the course of the project, quarantine restrictions were removed and work with the UK7 strain began. At this stage, many strain 5 mutants were through the first and second rounds of inoculation screening. The UK7 mutants were included in the study as a supplementary source of mutants. Considering the time pressure experienced at the latter stages of the study, these UK7 mutants could have been excluded from this study to reduce screening, PCR walking and sequencing time.

7.4.2 Future Studies

This study successfully identified 10 non-pathogenic mutants. Furthermore, four putative genes were identified from the flanking sequence of six of the non-pathogenic mutants. Functional characterisation of the four putative pathogenicity genes is yet to be performed and remains the highest priority task to be performed. To do this, it is first necessary to establish a reliable and efficient HR method. Once function is established, expression studies would be used to study gene function during pathogenesis.

7.4.2.1 Homologous Recombination Improvement

Considering the interest in *R. secalis* as a fungal pathogen and its continuing use as a laboratory fungus, experimental work focussed on enhancing HR efficiency would be beneficial. As previously mentioned (section 7.4.1) the length of flanking sequence that can successfully induce a HR event varies in different fungi and throughout the literature. Given this variation, a systematic study that directly compares different lengths of flanking sequence and the efficiency of HR could be useful in *R. secalis* to determine if HR can be optimised relative to the length of flanking sequence in knock out vectors. To make an accurate determination of the importance of flanking sequence in HR efficiency, it would be necessary to carry out these experiments using several different homologous nucleic acid sequences and with differing compositions to determine whether HR efficiency is sequence dependant.

Another method that could potentially improve HR efficiency in *R. secalis* is the use of a two-step selection system. By employing both positive and negative selection during HR, the amount of screening required to identify homologous and non-homologous integration events could be reduced. For example, efficiency of gene targeting in rice was enhanced by using both positive and negative selection. The targeting vector contained greater than 6 kb of homologous sequence on each flank, the hygromycin antibiotic gene as positive selection and the diphtheria toxin gene, located at the outside borders of the homologous sequence, as negative selection (Terada *et al.*, 2002). A similar vector structure could be used in *R. secalis* where, for example, the disruptive hygromycin resistance gene could select for transformants (positive selection) and green florescent

protein (GFP), located on the outside borders of the homologous flanking sequence, could identify the type of integration event (negative selection). Hence, non-homologous and homologous recombinants would be identified through the presence or absence of mutant florescence, respectively.

Targeted gene disruption through HR is used in a wide variety of organisms and given the number of genome sequences that have recently been completed and annotated, an efficient process that can identify a genes biological function is enthusiastically sought (Puchta, 2002). Using *S. cerevisiae* as a model it was shown that a number of genes are required for non-homologous T-DNA integration (van Attikum *et al.*, 2001). Identification of the genes controlling homologous and non-homologous recombination may provide an avenue to increase the efficiency of homologous recombination in a number of different species. With the complete genome of *N. crassa* sequenced, many putative genes were identified with no known function. To help deduce the gene functions an efficient high-throughput knockout procedure was established. This was successfully achieved by using the combination of recombination cloning in yeast and mutated *N. crassa* strains (Colot *et al.*, 2006). Recombination cloning in yeast bypasses the digestion-ligation steps in traditional cloning. The selected *N. crassa* strains had mutations of two genes, *mus-51* and *mus-52*, that are required for non-homologous end-joining (NHEJ). In the absence of these genes the efficiency of integrations by homologous recombination is enhanced (Ninomiya *et al.*, 2004). Studies in *Magnaporthe grisea* improved the efficiency of homologous recombination by identifying and inactivating the *MgKU80* gene, orthologous to the *mus-52* gene in *N. crassa* (Villalba *et al.*, 2008). Disruption of NHEJ in *R. secalis* could improve HR efficiency. Mutant strains of the fungus could be generated that have the homologues of the *mus-51* and *mus-52* genes disrupted to determine if they have a significant effect on the frequency of HR. If these mutants improved HR efficiency, they would become a powerful tool for the functional analysis of genes in *R. secalis*.

The importance of an efficient HR protocol in *R. secalis* grows as an increasing number of pathogenicity genes are identified in plant-pathogenic fungi. These and further potential expressed sequence tag (EST) candidates identified by microarray studies in pathogenic filamentous fungi (see review, Breakspear and Momany, 2007) could be rapidly tested for roles in *R. secalis* pathogenicity if an efficient HR methodology was available.

7.4.2.2 Expression Studies

Initially, amplification based strategies were considered to study the putative pathogenicity gene's functions. Reverse transcriptase-PCR (RT-PCR) is one method to compare putative gene ORF expression between mutant and wild type fungi. A fundamental failing of RT-PCR, however, is that no proof of the putative genes involvement in pathogenicity would be gained. Furthermore, the interpretation of RT-PCR results would be complicated because the expression of pathogenicity genes are often triggered or regulated during infection and colonisation of host tissue. The *ABC1* gene in *Magnaporthe grisea* was disrupted by insertional mutation in the gene's promoter. The subsequent reduction in the gene's expression demonstrated that the fungal pathogen required the up-regulation of the ABC transporter for pathogenesis (Urban *et al.*, 1999). Genes responsible for the pathogenicity of *Ustilago maydis* are regulated and, specifically, induced in infected host tissue (Kamper *et al.*, 2006). Two genes associated with pathogenicity were significantly up-regulated *in planta* during *Botrytis cinerea* infection of *Arabidopsis thaliana* (Goiti *et al.*, 2006). These examples suggest that gene regulation would make it difficult to predict the putative *R. secalis* pathogenicity genes expression. Furthermore, the location of plasmid integration in the putative gene's promoters would confound expression studies with the potential that expression is limited or possibly increased by promoter integration rather than completely inhibited.

A more detailed study of both mutant and wild type gene expression *in vitro* and *in planta* using quantitative PCR would provide strong evidence about the role of these genes in pathogenicity. Expression studies of a cluster of pathogenicity genes in the pea pathogen *Nectria haematococca* showed differing expression profiles for the genes located at the cluster (Liu *et al.*, 2003). However, this type of investigation would necessitate a major expression study detailing different stages of infection. To conclude, the potential information that could be yielded by amplification studies would only have significance after the putative genes role had been proven.

7.4.2.3 Fungal Pathogenicity: Current Perspectives

Successful colonization of a host by a fungal pathogen requires a number of highly specialised structures and the production of a suite of molecules that manipulate the host. Many of the genes involved in fungal pathogenicity of plants have been identified (Baldwin *et al.*, 2006). Jones and

Dangl (2006) provide a model of the complex molecular interaction between plant immune systems and the effector molecules secreted by pathogens that determine pathogen virulence. This model separates the immune system in plants into two branches: transmembrane pattern recognition receptors respond to slowly evolving microbial- or pathogen-associated molecular patterns (MAMPs and PAMPs) and; intracellular protein products encoded by the nucleotide binding site-leucine rich repeat (NBS-LRR) proteins that are encoded by most plant resistance genes (*R*). Effector molecules are diverse in both their structure and activity (see review, Kamoun, 2007) and are responsible for overcoming PAMP triggered immunity (Jones and Dangl, 2006). The evolution of *R* genes provides a mechanism for plants to detect pathogen effectors and trigger a cascade of defence responses that limit pathogen infection (see section 1.2.2 Resistance). Effector molecules are classified into two classes, apoplastic and cytoplasmic effectors, dependant on their target site in the host plant (Kamoun, 2006). Apoplastic effectors are secreted by the fungus into the plant extracellular space. For example, the Avr4 protein from *Cladosporium fulvum* binds to fungal cell walls and protects fungal cell walls from hydrolysis by plant chitinases (van den Burg *et al*., 2006). Cytoplasmic effectors are translocated into the plant cell. Virulence effector functions have been demonstrated for the cytoplasmic Avra10 and Avrk1 effector proteins of *Blumeria graminis* f sp *hordei* (Ridout *et al*., 2006). Considering plant pathogens are of major importance in agriculture and forestry, the study of their biology and interaction with host plants is a foundation stone of continued world food security (Ellis *et al.*, 2007). Hence, the study of fungal effector proteins, their evolution, mode of action and role in virulence are key questions for future research.

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Appendix A: PCR Oligonucleotides

All oligonucleotides are presented in 5′ to 3′.

General Oligonucleotides

Name	Sequence
HphNotIfwd	cggccgccccgcggccgcgaagtggaaagg
HphNotIrev	cttctgcttcgcggccgcctgaagggcgtac
Adaptor 1	ctaatacgactcactatagggctcgagcggccgcccggggaggt
Adaptor 2	acctcccc
AP1	ggatcctaatacgactcactatagggc
AP2	ctatcgggctcgagcggc
T7	taatacgactcactataggg
SP6	atttaggtgacactatag

Name	Sequence
PlaR1	acacaaatcgcccgcagaag
PlaR1.1	cagaagcgcggccgtctggaccg
PlaR2	gatcctgaacaccatttgtc
PlaR2.1	tgtctcaactccggagctgacatc
PlaR3	cctatgagtcgtttacccag
PlaR3.1	acccagaatgcacaggtacacttg
PlaR4	cctcttcgctattacgccag
PlaR4.1	gccagctggcgaaagggggatg

Mutant YB4.20 Oligonucleotides

Walking Primers

Name	Sequence
4.20 1	aaattgtgtggatgatgcttgaaaacagcc
4.20 1.1	cagccaacaatgcaatgaatctcaatcca
4.20 2	tgtatataaagtcaatttattggagagacg
4.20 2.1	aatgggcttgttaccaaagtcagaacacca

Name	Sequence
4.20 3	gaaaaatgtggcgtgaatactggcata
4.20 3.1	taagtcatcagctgaagggcagcgcg
4.20rw#1	cgacgctagtctatgcagggaacattc

PCR and Sequencing Primers

Name	Sequence
24.1a	ggctgaaatggctgtcctat
4.20check#1	gcaatgaccagatcagactgagtgacaacg
4.20check#2	ttggccttcctcctcgttacctcgtg
4.20check#3	gtgacaagcgcatgttgggttgagac
4.20check#4	gccttgcacacttgaaaagcagtcca
6.1a	cccacccaggtaagaatgttcaagcacaag
8.1a	ccttgtcctgtaaaaacctggtgggt
4.20fwd	attcgcccaccaaacaagcgagcacgcaga aaattg

Name	Sequence
4.20rev	cattccctcggctctctcaatactcgta
4.20seq#1	aagcgcatcttttccggctc
4.20seq#2	acgactactggcgaactcctccaa
4.20seq#3	ctccgcagttggggatagggtaca
4.20seq#4	gaaagctggaatacctcggcatagg
4.20seq#5	atccgctgttagttcctttcctctcc
4.20seq#6	caaccagggcattgtcgttatctctc

Mutant YB4.44 Oligonucleotides

Walking Primers

Name	Sequence
4.44 1	ccgagaaatcggctacaaatgatacca
4.44 1.1	tcgaaagcttcagatatgggttccttagcc
4.44 2	tgccaatgaccagttttacgaacagaacga
4.44 2.1	atatccaattctgacatttgagcgag
4.44 3	cggacttgggagactatctgaagtttcacg

Name	Sequence
4.44 3.1	acaaggaagggcgagcccctcaatagttta
4.44 4	aacgaagtccatcgctttgtagacgagc
4.44 4.1	cgaaagcgaatcttcaaagccgaacc
4.44 5	cctcctgtttcggaagagtatatgggca
4.44 5.1	tacagacacattggtcgccactgtca

PCR and Sequencing Primers

Name	Sequence
4.44check#1	tgatcctccagcttcccagc
4.44check#2	cgtgaaagacatcgtgacac
4.44 check#3	gctgttgtaatggctccgatgaatcc
4.44 check #4	cgaccatgttgaacatcgagtaaggg
4.44check#5	tcacgttattacttcttttgacagctgctt

Name	Sequence
4.44check#6	cgcgggaaaactatttggcaaat
4.44Sbffwd	cctgcagg.gggtactacgctcattttttgaatag catt
4.44Xmarev	cccggg.ttaccgttgtggacggagattactag
4.44seq#1	aaacctaacaaccacatacgccgtctcc
4.44seq#3	gaaggtaagtctcgacttgttttcaggggc

Mutant YB7.395 Oligonucleotides

Walking Primers

Name	Sequence
7.395 1	tgacgggaggtcccttatcgcggata
7.395 1.1	gcggatagagagatctgagtttggaa
7.395 2	ccgaccaatcaggcttgcttagttccaaac
7.395 2.1	tatccgcgataagggacctcccgtca
7.395 3	cgccgaatcctgcaatcatcttttcc

Name	Sequence
7.395 3.1	tacaagatacctgggtccgcatttcc
7.395 4	cgcactgacacttctgttgcagtattcacc
7.395 4.1	gcaatccacaaggctcgatcgcacac

PCR and Sequencing Primers

Name	Sequence
9.4a	aaacctctccatcaaccccctacactcctc
7.395check#1	cattgtaatactcgctcgctctctctcc
7.395check#2	ttggtgagtaatgtgatgaggataggtacc
3.1a	tgcttagcaaatgcccgccc
63.4a	ggtcatctgctcgaagtgcgatggga

Name	Sequence
7.395fwdHind	aagctt..ccgaggctgtcaagattagaggga ac
7.395seq#1	ggaagagcttgagatcttgttgtctcgg
7.395seq#2	tcattcatttttgagatgatgtgattgtgg

Mutant YB7.412 Oligonucleotides

Walking Primers

Name	Sequence
7.412 1	attcttgaccctagctaggcctgctcccc
7.412 1.1	tcttacccaccccgtctggattcacc
7.412 2	gctgtatatagcgaatccggatgcgg
7.412 2.1	gcaggcaggcaggcaaaggaggatat
7.412 3	ggatcacgacgagaaagaaaggagatgg
7.412 3.1	ggaaaggagaggagagaagaggaaagg

Name	Sequence
7.412 4	ttaaatgaacggagatacttacatcctggg
7.412 4.1	gatccagacgacagcgctacgatgat
7.412 5	atatgcgtcaaaacaaatatacctggctca
7.412 5.1	catgcgatttttcactcgtcttctcggc
7.412 6	accaaccaactcagtttgatcacgtacgg
7.412 6.1	aacggtactatcgattagtatccaccttgc

PCR and Sequencing Primers

Name	Sequence
7.412check#1	gagagcagaggaggaggaaa
7.412check#2	gagatcttgagatgtttggcggtagg
7.412check#3	cgtcaaccacgaacgatagcaaggta
7.412check#4	agggttagagagtttaggtgaggcgtg
7.412check#5	gtcctgaacaggaatcttggtcttgg
7.412fwdSbf	cctgcagg..ggcgaaggaagatcactgtagg ttga
7.412revXma	cccggg..gtccccgtctacaccatgccatgtgt

Name	Sequence
7.412seq#1	tgcctgcctgcccctctagtcactat
7.412seq#2	ttgcctgaactccatctcccacccgt
7.412seq#3	cgtattcgctggggttacgtttcgtgtttc
7.412seq#4	ctgccatgttggattgagtgccgtca
7.412a	tcagaaaggaagcccagctgagg
7.412b	tctcaagatctccgccgctcgacgt

Mutant YH4.5 Oligonucleotides

Walking Primers

Name	Sequence
4.5 1	ccagcatctcccacaatcccaataca
4.5 1.1	atccagaactcaagcagcaaagcccg
4.5 2	gctggagcaattgacgcgtgtggtct
4.5 2.1	ggttgacacagtcgcagaaaactgaagc

Name	Sequence
4.5 3	gcagatcgtgttgttaacagatttggagat
4.5 3.1	ccttcacgggctttcatcagctagct
4.5 4	agtactcctactagagctgctcgaactgcg
4.5 4.1	aagtctttcctgccgtacctctgctag

PCR and Sequencing Primers

Name	Sequence
64.3 a	ctaaacttcccaaagctcagaaca
64.3b	caactgtcataacgcagtggtgag
81.1a	tcggcagaggcgtatttaggctg
4.5check#1	gcatctaggatagtttctccagtggtgc
4.5rw#1	cacaagcaactgccgatatttccg

Name	Sequence
4.5SbfIfwd	cctgcagg..ttgttgtctttgcgttggtgcttgcg
4.5SbfIrev	cctgcagg.. catttgaggccttaaccgcatcga
4.5seq#1	aaatccgtaagtccccgtcgagtc
4.5seq#2	agcgaaaaacgctaaaaccgcg

Mutant LH2013 Oligonucleotides

Walking Primers

Name	Sequence
2013 1	atgtgagggcccatggcttatcctgg
2013 1.1	ccctcttggcctatccatgcattattcg
2013 2	cctgtgcgaaagcacggagagcctag
2013 2.1	gctcgtggggttctcactgcagtatg
2013 3	ctatccagcactttgcgcccttctt
2013 3.1	cgatctttgacattccttttcctcgc
2013 4	tatgacagggccacttcctcgtcggag
2013 4.1	ggacacaaatggtccaaaagaggagc

Name	Sequence
2013 5	cctcccctggtgctctttaaggttatgt
2013 5.1	gattcgatacgatgctagcctccaca
2013 6	cccacctaccaagaccacgataagga
2013 6.1	agacagcatccaccgtcgtccatcca
2013 7	tgctgtctccagtatggtgccgtaag
2013 7.1	cgtaagatcatgtcttggatttgtggct

PCR and Sequencing Primers

Name	Sequence
2013check#1	cgtccaattgccgcgatcaa
2013check#1a	tctccgtgctttcgcacaggattaag
2013check#2	aagaacgccgaccggaactctctacg
2013check#3	ggagccaatggaatccatgatcagatcg
2013check#4	ccgagctagggctaagcttgatcactgata
87.1a	cttgagtccttagtttttgttttgctgacc

Name	Sequence
2013Bamfwd	ggatcc..tcgacataggagagcataccgacc tt
2013Sacrev	gagctc..tcgtctgctcaatctccagtgccatt
2013seq#1	accaccctgcagcgacgaggaagaa
2013seq#2	accgcagaagatagcaagaaccaagagag g

Oligonucleotides used in KO vector Construction

Name	Sequence
hphBamHIfwd	ggatcc..ccttgtatctctacacacaggctca
Hygroprbrev	cggagctgacatcgacacc

Name	Sequence
HphEcoRVfwd	gatatc..ccttgtatctctacacacaggctca
HphEcoRVrev	gatatc..gggcgtactagggttgcgaggtc

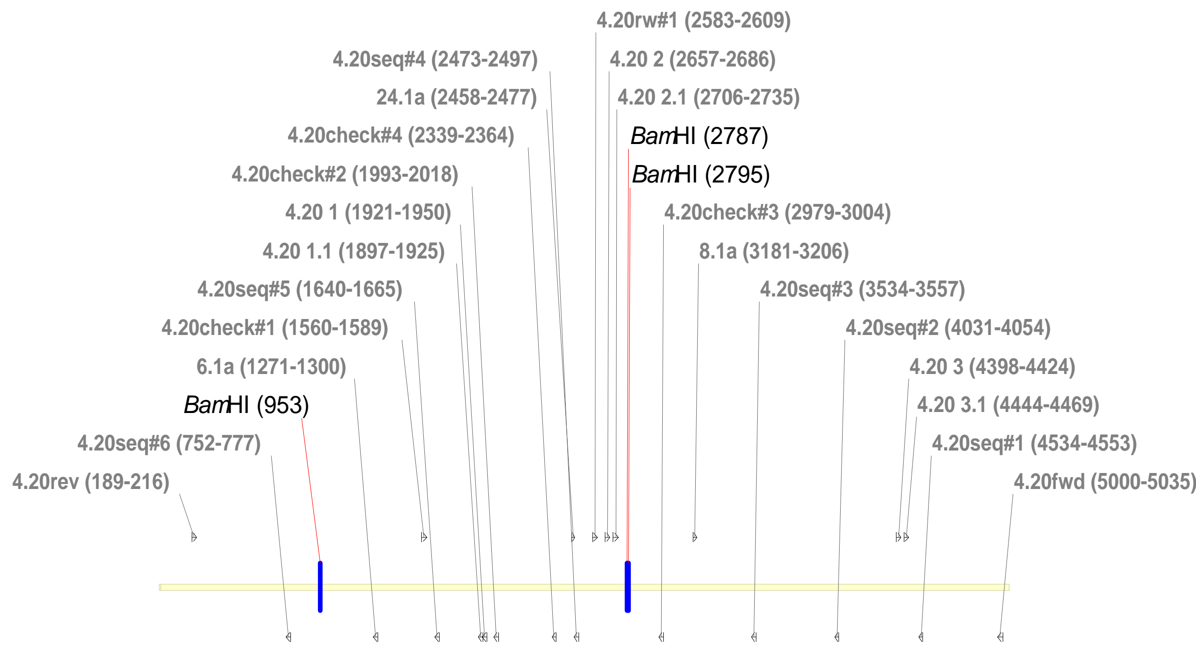
Oligonucleotides used in Screening for Homologous Recombination

Name	Sequence
4.44hph	ggctccaacaatgtcctgacgga
4.44 KO 1	ggtcccaattttccagttggaggtcttcac
4.44 KO 2	acgctaagagtttagtatcggctgctgagc

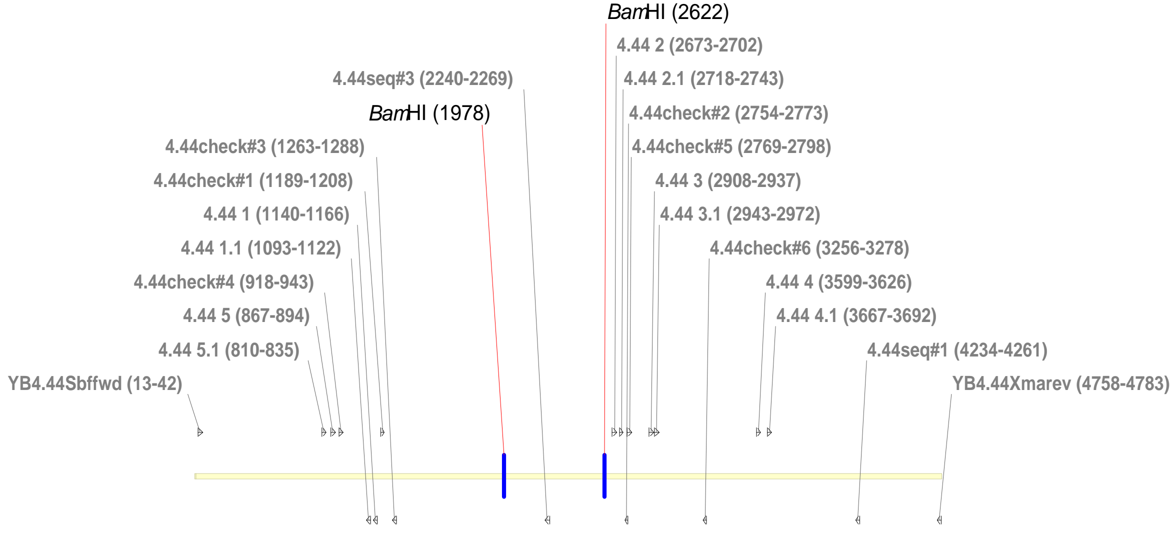
Name	Sequence
2013 hph	atatgctccgcattggtcttgacc
2013 KO check	gttgacagaccgtatggaatagatgcgagc
2013 KO 1	tatcctcgactcccgccctgtctt
2013 KO 2	ccccaaagaacggttgtaggtcca

Appendix B: PCR Oligonucleotides - Location

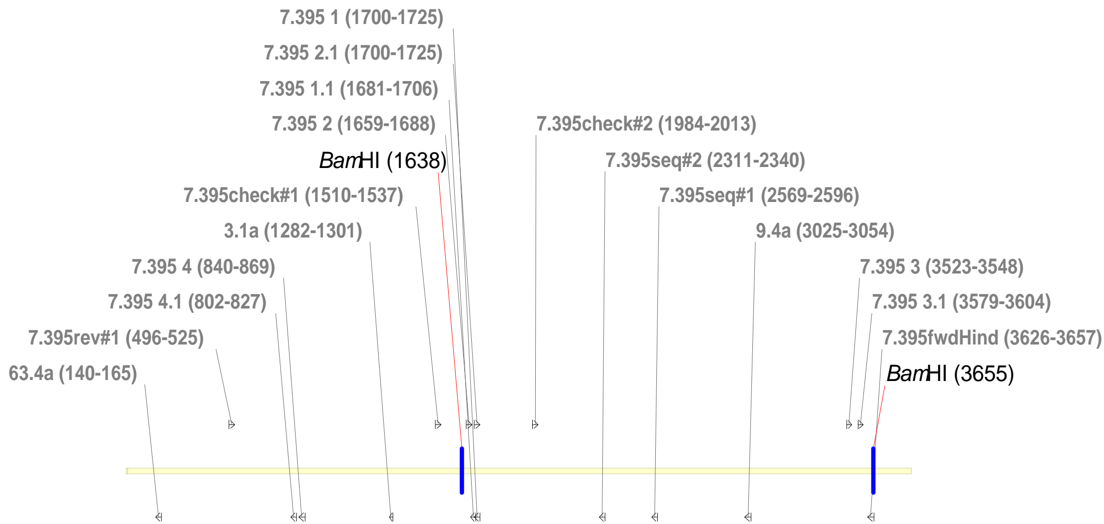
YB4.20



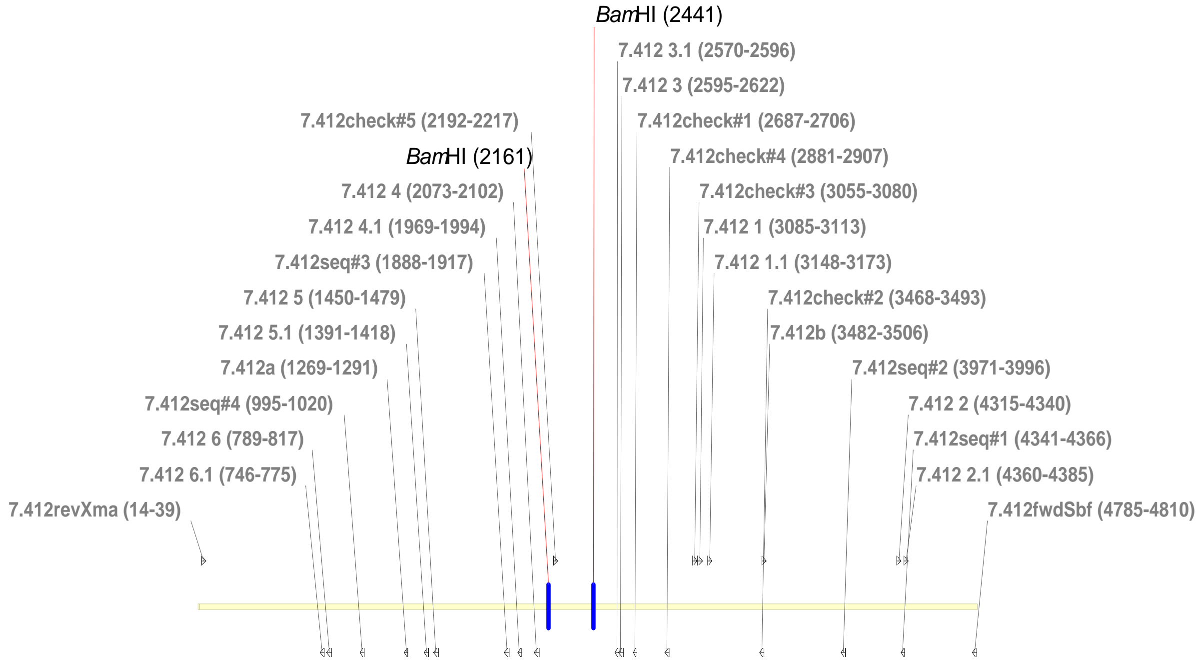
YB4.44



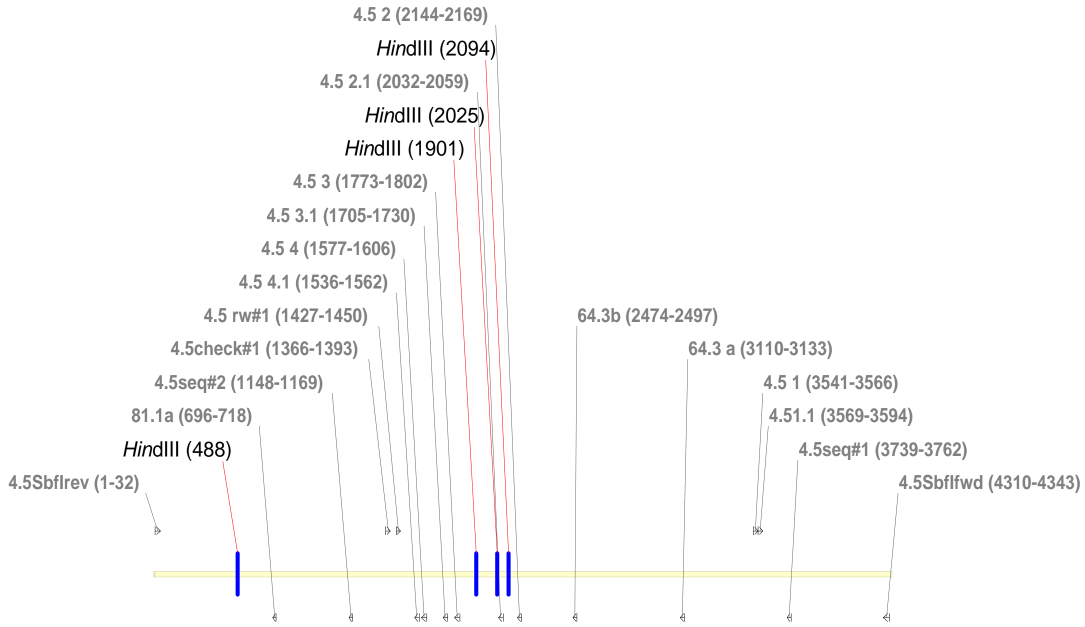
YB7.395



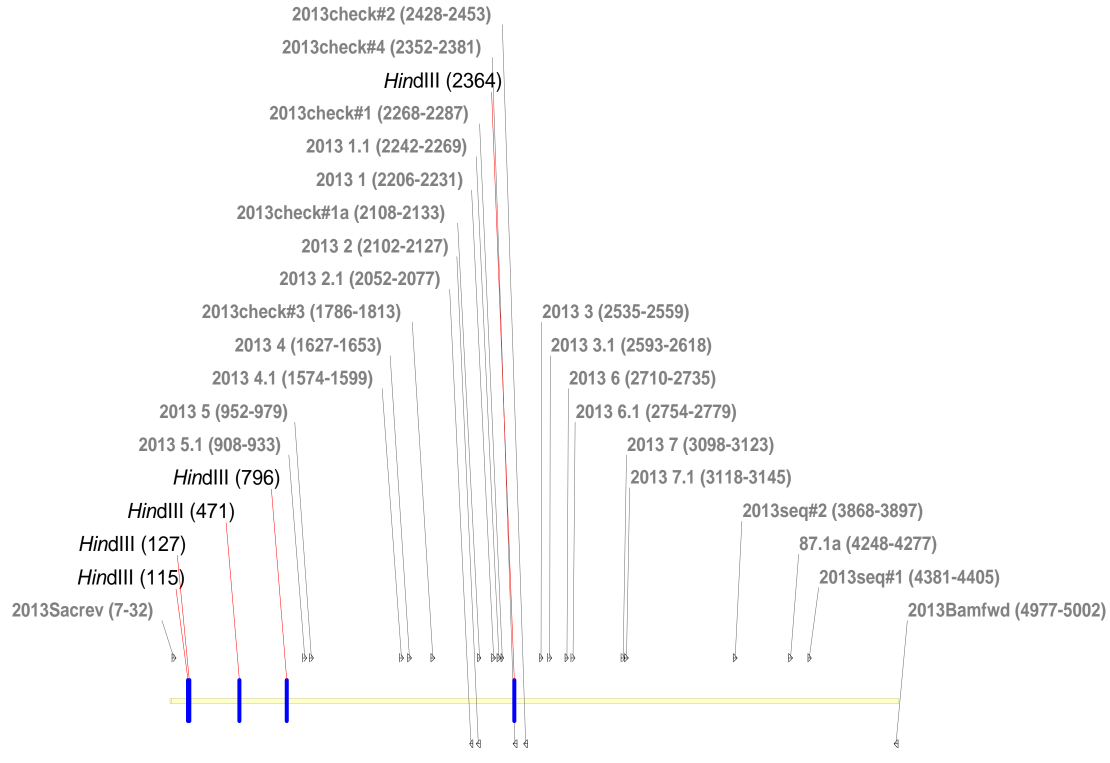
YB7.412



YH4.5

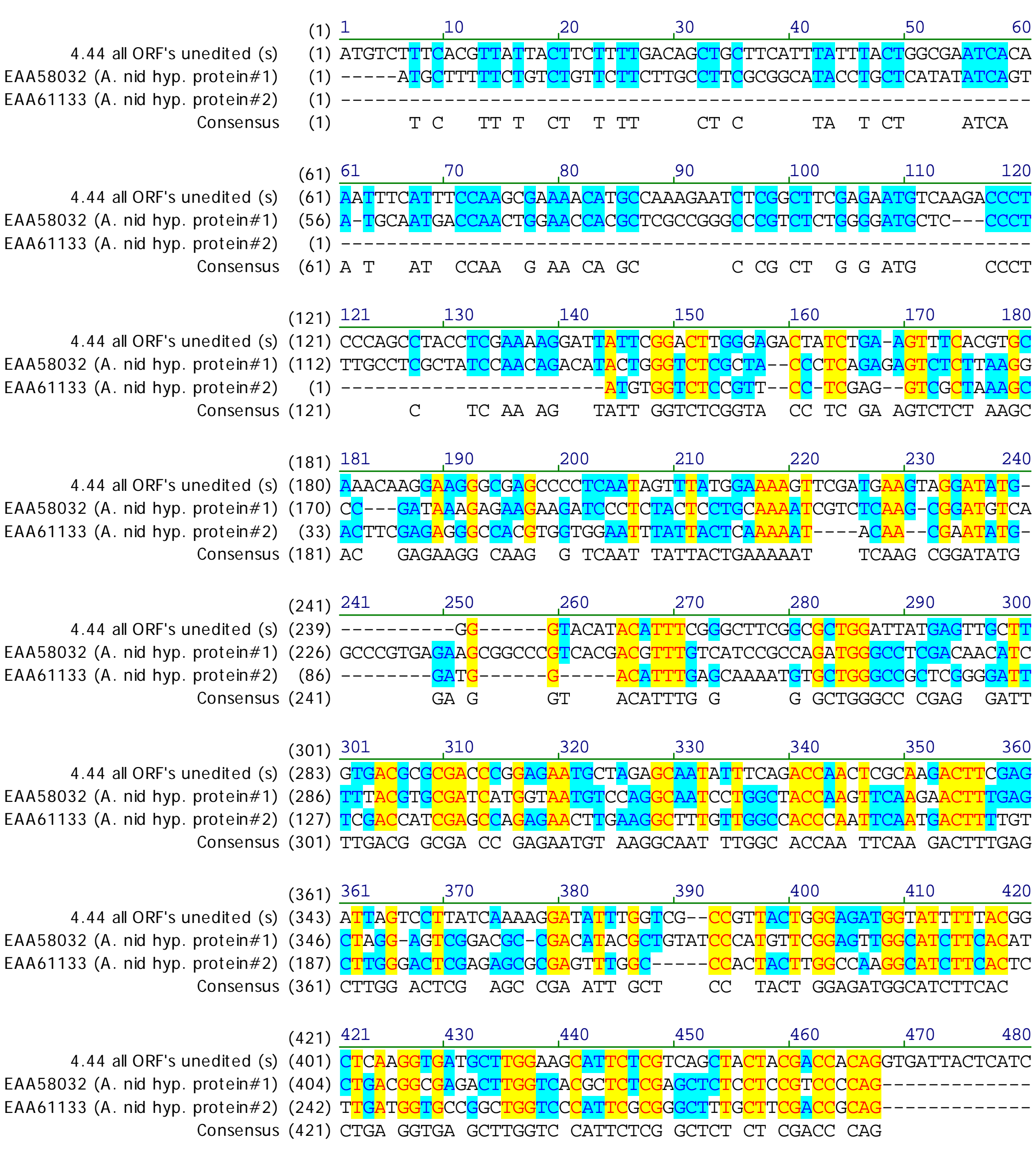


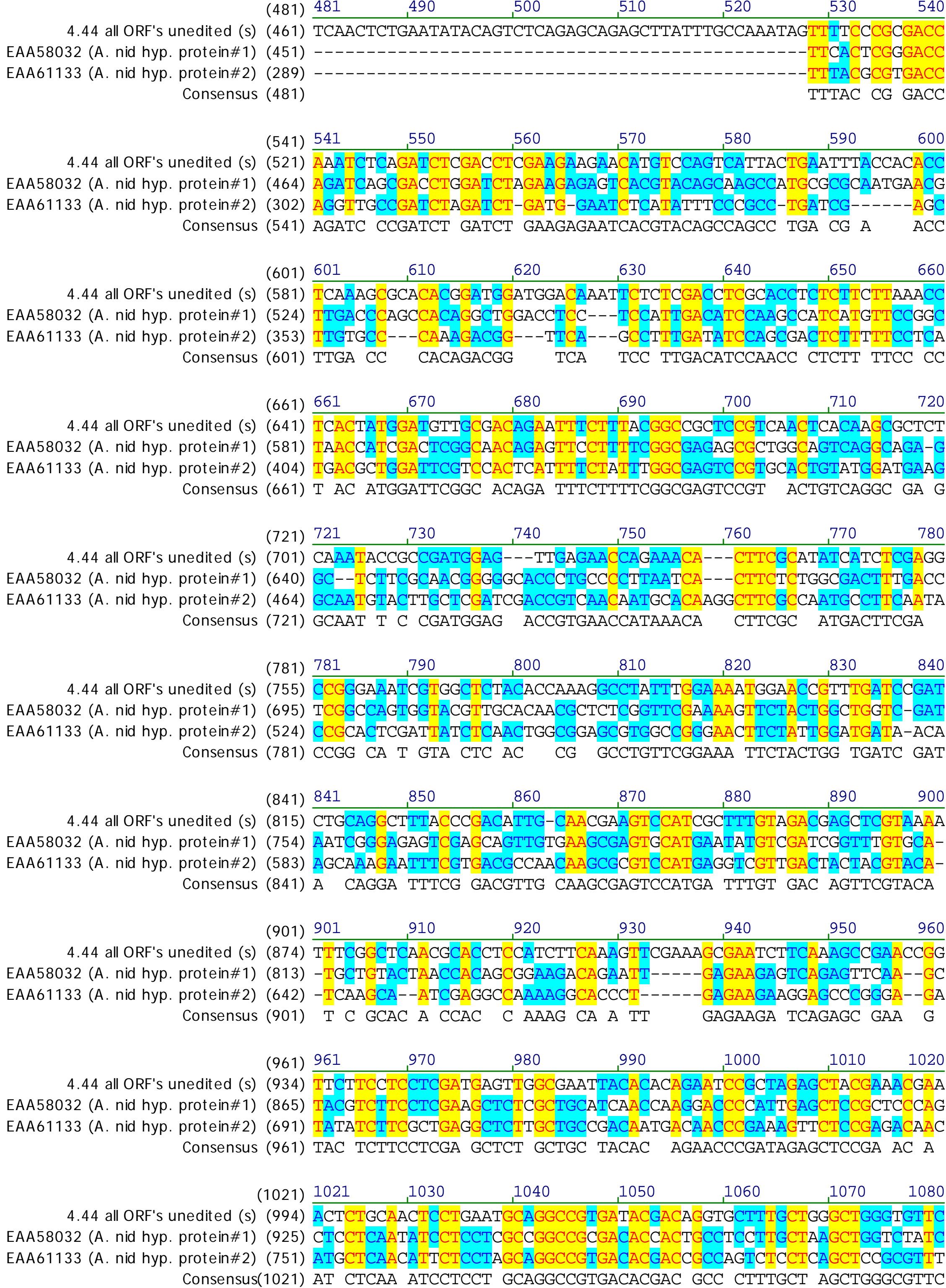
LH2013

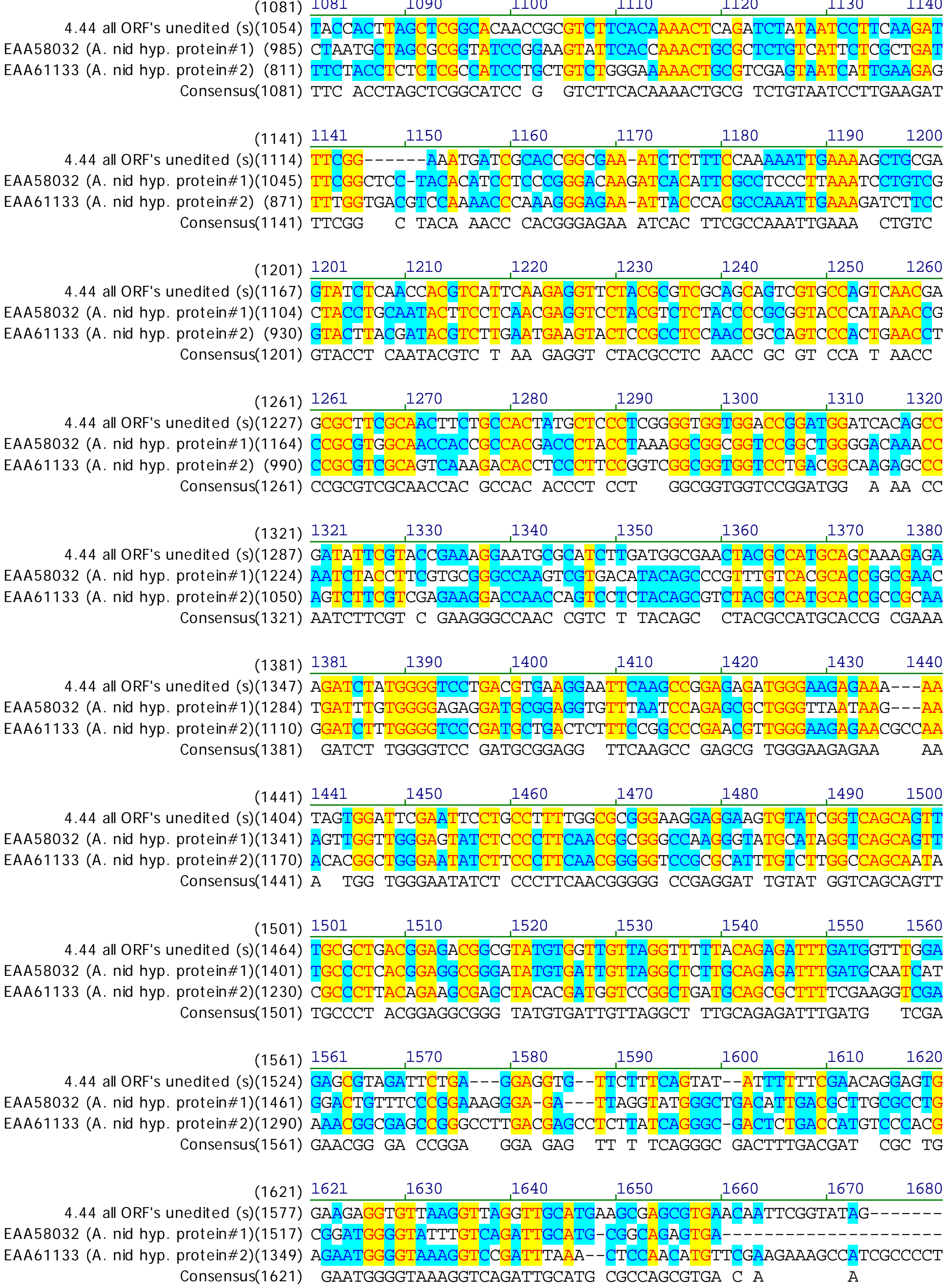


Appendix C: DNA Alignments

Nucleic acid sequence of mutant YB4.44 ORFs of interest with DNA sequence of two hypothetical *A. nidulans* proteins (accession numbers: EAA58032 and EAA61133).



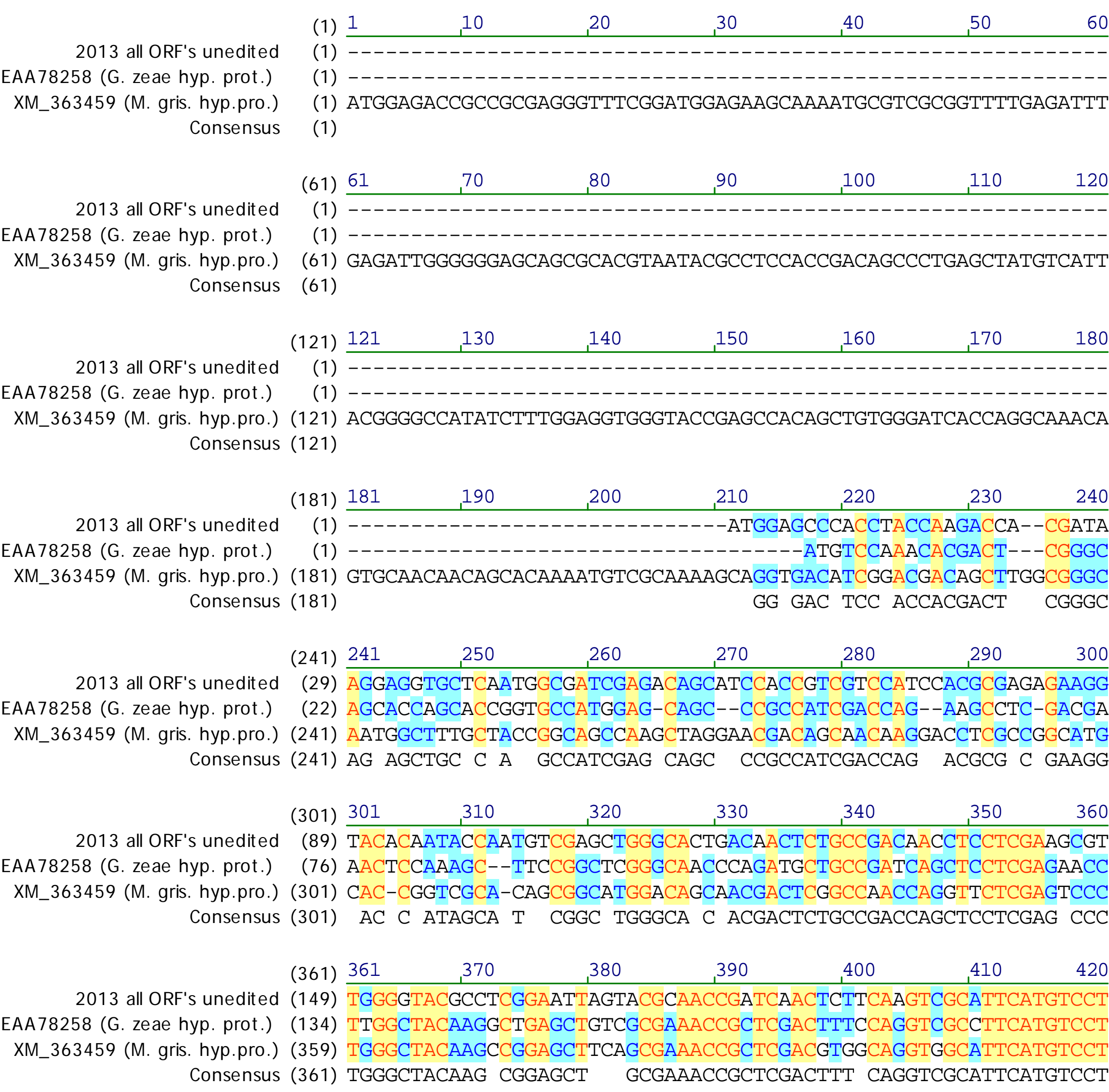


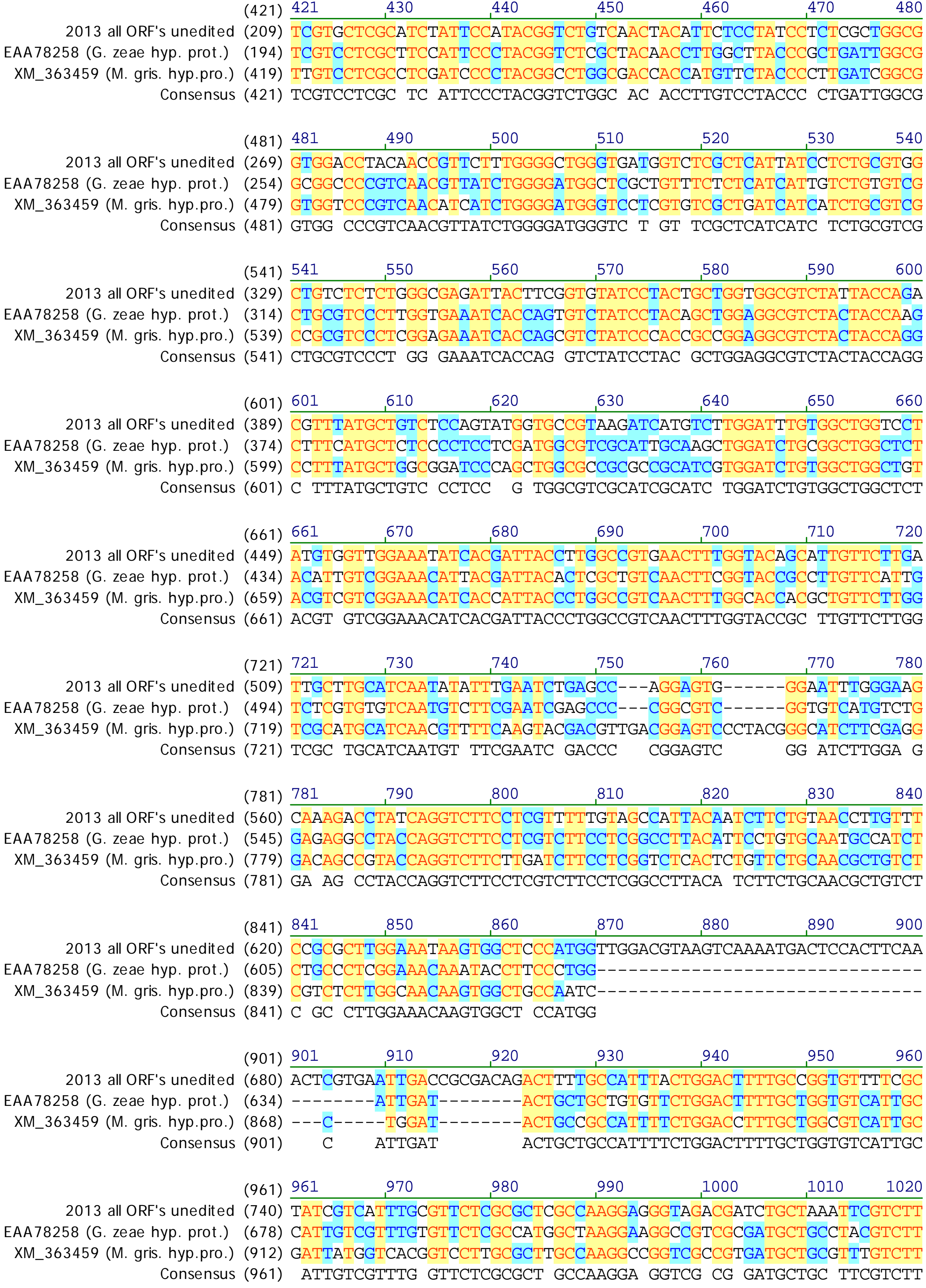


(1081) 1081 1090 1100 1110 1120 1130 1140
4.44 all ORF's unedited (s)(1054) TACCACTTAGCTCGGCACAACCGCGTCTTCACAAAACTCAGATCTATAATCCTTCAAGAT
EAA58032 (A. nid hyp. protein#1) (985) CTAATGCTAGCGCGGTATCCGGAAGTATTCACCAAACTGCGCTCTGTCATTCTCGCTGAT
EAA61133 (A. nid hyp. protein#2) (811) TTCTACCTCTCTCGCCATCCTGCTGTCTGGGAAAAACTGCGTCGAGTAATCATTGAAGAG
Consensus(1081) TTC ACCTAGCTCGGCATCC G GTCTTCACAAAACTGCG TCTGTAATCCTTGAAGAT
(1141) 1141 1150 1160 1170 1180 1190 1200
4.44 all ORF's unedited (s)(1114) TTCGG------AAATGATCGCACCGGCGAA-ATCTCTTTCCAAAAATTGAAAAGCTGCGA
EAA58032 (A. nid hyp. protein#1)(1045) TTCGGCTCC-TACACATCCTCCCGGGACAAGATCACATTCGCCTCCCTTAAATCCTGTCG
EAA61133 (A. nid hyp. protein#2) (871) TTTGGTGACGTCCAAAACCCAAAGGGAGAA-ATTACCCACGCCAAATTGAAAGATCTTCC
Consensus(1141) TTCGG C TACA AACC CACGGGAGAA ATCAC TTCGCCAAATTGAAA CTGTC
(1201) 1201 1210 1220 1230 1240 1250 1260
4.44 all ORF's unedited (s)(1167) GTATCTCAACCACGTCATTCAAGAGGTTCTACGCGTCGCAGCAGTCGTGCCAGTCAACGA
EAA58032 (A. nid hyp. protein#1)(1104) CTACCTGCAATACTTCCTCAACGAGGTCCTACGTCTCTACCCCGCGGTACCCATAAACCG
EAA61133 (A. nid hyp. protein#2) (930) GTACTTACGATACGTCTTGAATGAAGTACTCCGCCTCCAACCGCCAGTCCCACTGAACCT
Consensus(1201) GTACCT CAATACGTC T AA GAGGT CTACGCCTC AACC GC GT CCA T AACC
(1261) 1261 1270 1280 1290 1300 1310 1320
4.44 all ORF's unedited (s)(1227) GCGCTTCGCAACTTCTGCCACTATGCTCCCTCGGGGTGGTGGACCGGATGGATCACAGCC
EAA58032 (A. nid hyp. protein#1)(1164) CCGCGTGGCAACCACCGCCACGACCCTACCTAAAGGCGGCGGTCCGGCTGGGGACAAACC
EAA61133 (A. nid hyp. protein#2) (990) CCGCGTCGCAGTCAAAGACACCTCCCTTCCGGTCGGCGGTGGTCCTGACGGCAAGAGCCC
Consensus(1261) CCGCGTCGCAACCAC GCCAC ACCCT CCT GGCGGTGGTCCGGATGG A AA CC
(1321) 1321 1330 1340 1350 1360 1370 1380
4.44 all ORF's unedited (s)(1287) GATATTCGTACCGAAAGGAATGCGCATCTTGATGGCGAACTACGCCATGCAGCAAAGAGA
EAA58032 (A. nid hyp. protein#1)(1224) AATCTACCTTCGTGCGGGCCAAGTCGTGACATACAGCCCGTTGTCACGCACCGGCGAAC
EAA61133 (A. nid hyp. protein#2)(1050) AGTCTTCGTCGAGAAGGACCAACCAGTCCTCTACAGCGTCTACGCCATGCACCGCCGCAA
Consensus(1321) AATCTTCGT C GAAGGGCCAAC CGTC T TACAGC CTACGCCATGCACCG CGAAA
(1381) 1381 1390 1400 1410 1420 1430 1440
4.44 all ORF's unedited (s)(1347) AGATCTATGGGGTCCTGACGTGAAGGAATTCAAGCCGGAGAGATGGGAAGAGAAA---AA
EAA58032 (A. nid hyp. protein#1)(1284) TGATTTGTGGGGAGAGGATGCGGAGGTGTTTAATCCAGAGCGCTGGGTTAATAAG---AA
EAA61133 (A. nid hyp. protein#2)(1110) GGATCTTTGGGGTCCCGATGCTGACTCTTTCCGGCCCGAACGTTGGGAAGAGAACGCCAA
Consensus(1381) GATCT TGGGGTCC GATGCGGAGG TTCAAGCC GAGCG TGGGAAGAGAA AA
(1441) 1441 1450 1460 1470 1480 1490 1500
4.44 all ORF's unedited (s)(1404) TAGTGGATTCGAATTCCTGCCTTTTGGCGCGGGAAGGAGGAAGTGTATCGGTCAGCAGTT
EAA58032 (A. nid hyp. protein#1)(1341) AGTTGGTTGGAGTATCTCCCCTTCAACGGCGGGCCAAGGGTATGCATAGGTCAGCAGTT
EAA61133 (A. nid hyp. protein#2)(1170) ACACGGCTGGGAATATCTTCCCTTCAACGGGGGTCCGCGCATTTGTCTTGGCCAGCAATA
Consensus(1441) A TGG TGGGAATATCT CCCTTCAACGGGGG CCGAGGAT TGTAT GGTCAGCAGTT
(1501) 1501 1510 1520 1530 1540 1550 1560
4.44 all ORF's unedited (s)(1464) TGCGCTGACGGAGACGGCGTATGTGGTTGTTAGGTTTTTACAGAGATTTGATGGTTTGGA
EAA58032 (A. nid hyp. protein#1)(1401) TGCCCTCACGGAGGCGGGATATGTGATTGTTAGGCTCTTGCAGAGATTTGATGCAATCAT
EAA61133 (A. nid hyp. protein#2)(1230) CGCCCTTACAGAAGCGAGCTACACGATGGTCCGGCTGATGCAGCGCTTTTCGAAGGTCGA
Consensus(1501) TGCCCT ACGGAGGCGGG TATGTGATTGTTAGGCT TTGCAGAGATTTGATG TCGA
(1561) 1561 1570 1580 1590 1600 1610 1620
4.44 all ORF's unedited (s)(1524) GAGCGTAGATTCTGA---GGAGGTG--TTCTTTCAGTAT--ATTTTTCGAACAGGAGTG
EAA58032 (A. nid hyp. protein#1)(1461) GGACTGTTTCCCGGAAAGGGA-GA---TTAGGTATGGGCTGACATTGACGCTTGCGCCTG
EAA61133 (A. nid hyp. protein#2)(1290) AAACGGCGAGCCGGGCCTTGACGAGCCTCTTATCAGGGC-GACTCTGACCATGTCCCACG
Consensus(1561) GAACGG GA CCGGA GGA GAG TT T TCAGGGC GACTTTGACGAT CGC TG
(1621) 1621 1630 1640 1650 1660 1670 1680
4.44 all ORF's unedited (s)(1577) GAAGAGGTGTTAAGGTTAGGTTGCATGAAGCGAGCGTGAACAATTCGGTATAG-------
EAA58032 (A. nid hyp. protein#1)(1517) CGGATGGGGTATTTGTCAGATTGCATG-CGGCAGAGTGA---------------------
EAA61133 (A. nid hyp. protein#2)(1349) AGAATGGGGTAAAGGTCCGATTTAAA--CTCCAACATGTTCGAAGAAAGCCATCGCCCCT
Consensus(1621) GAATGGGGTAAAGGTCAGATTGCATG CGCCAGCGTGA C A A

(1681) 1681 1699
4.44 all ORF's unedited (s)(1630) -------------------
EAA58032 (A. nid hyp. protein#1)(1555) -------------------
EAA61133 (A. nid hyp. protein#2)(1407) CCAAACATGCACACGTTAA
Consensus(1681)

Nucleic acid sequence of mutant LH2013 ORFs of interest with DNA sequence of two hypothetical proteins from *G. zeae* (accession number: EAA78258) and *M. grisea* (accession number: XM_363459).




(421) 421 430 440 450 460 470 480
2013 all ORF's unedited (209) TCGTGCTCGCATCTATTCCATACGGTCTGTCAACTACATTCTCCTATCCTCTCGCTGGCG
EAA78258 (G. zeae hyp. prot.) (194) TCGTCCTCGCTTCCATTCCCTACGGTCTCGCTACAACCTTGGCTTACCCGCTGATTGGCG
XM_363459 (M. gris. hyp.pro.) (419) TTGTCCTCGCCTCGATCCCCTACGGCCTGGCGACCACCATGTTCTACCCCTTGATCGGCG
Consensus (421) TCGTCCTCGC TC ATTCCCTACGGTCTGGC AC ACCTTGTCCTACCC CTGATTGGCG
(481) 481 490 500 510 520 530 540
2013 all ORF's unedited (269) GTGGACCTACAACCGTTCTTGGGGCTGGGTGATGGTCTCGCTCATTATCCTCTGCGTGG
EAA78258 (G. zeae hyp. prot.) (254) GCGGCCCCGTCAACGTTATCTGGGGATGGCTCGCTGTTTCTCTCATCATTGTCTGTGTCG
XM_363459 (M. gris. hyp.pro.) (479) GTGGTCCCGTCAACATCATCTGGGGATGGGTCCTCGTGTCGCTGATCATCATCTGCGTCG
Consensus (481) GTGG CCCGTCAACGTTATCTGGGGATGGGTC T GT TCGCTCATCATC TCTGCGTCG
(541) 541 550 560 570 580 590 600
2013 all ORF's unedited (329) CTGTCTCTCTGGGCGAGATTACTTCGGTGTATCCTACTGCTGGTGGCGTCTATTACCAGA
EAA78258 (G. zeae hyp. prot.) (314) CTGCGTCCCTTGGTGAAATCACCAGTGTCTATCCTACAGCTGGAGGCGTCTACTACCAAG
XM_363459 (M. gris. hyp.pro.) (539) CCGCGTCCCTCGGAGAAATCACCAGCGTCTATCCCACCGCCGGAGGCGTCTACTACCAGG
Consensus (541) CTGCGTCCCT GG GAAATCACCAG GTCTATCCTAC GCTGGAGGCGTCTACTACCAGG
(601) 601 610 620 630 640 650 660
2013 all ORF's unedited (389) CGTTTATGCTGTCTCCAGTATGGTGCCGTAAGATCATGTCTTGGATTTGTGGCTGGTCCT
EAA78258 (G. zeae hyp. prot.) (374) CTTTCATGCTCTCCCCTCCTCGATGGCGTCGCATTGCAAGCTGGATCTGCGGCTGGCTCT
XM_363459 (M. gris. hyp.pro.) (599) CCTTTATGCTGGCGGATCCCAGCTGGCGCCGCGCCGCATCGTGGATCTGTGGCTGGCTGT
Consensus (601) C TTTATGCTGTC CCTCC G TGGCGTCGCATCGCATC TGGATCTGTGGCTGGCTCT
(661) 661 670 680 690 700 710 720
2013 all ORF's unedited (449) ATGTGGTTGGAAATATCACGATTACCTTGGCCGTGAACTTTGGTACAGCATTGTTCTTGA
EAA78258 (G. zeae hyp. prot.) (434) ACATTGTCGGAAACATTACGATTACACTCGCTGTCAACTTCGGTACCGCCTTGTTCATTG
XM_363459 (M. gris. hyp.pro.) (659) ACGTCGTCGGAAACATCACCATTACCCTGGCCGTCAACTTTGGCACCACGCTGTTCTTGG
Consensus (661) ACGT GTCGGAAACATCACGATTACCCTGGCCGTCAACTTTGGTACCGC TTGTTCTTGG
(721) 721 730 740 750 760 770 780
2013 all ORF's unedited (509) TTGCTTGCATCAATATATTTGAATCTGAGCC---AGGAGTG------GGAATTTGGAAG
EAA78258 (G. zeae hyp. prot.) (494) TCTCGTGTGTCAATGTCTTCGAATCGAGCCC---CGGCGTC------GGTGTCATGTCTG
XM_363459 (M. gris. hyp.pro.) (719) TCGCATGCATCAACGTTTTCAAGTACGACGTTGACGGAGTCCCTACGGGCATCTTCGAGG
Consensus (721) TCGC TGCATCAATGT TTCGAATC GACCC CGGAGTC GG ATCTTGGA G
(781) 781 790 800 810 820 830 840
2013 all ORF's unedited (560) CAAAGACCTATCAGGTCTTCCTCGTTTTTGTAGCCATTACAATCTTCTGTAACCTTGTTT
EAA78258 (G. zeae hyp. prot.) (545) GAGAGGCCTACCAGGTCTTCCTCGTCTTCCTCGGCCTTACATTCCTGTGCAATGCCATCT
XM_363459 (M. gris. hyp.pro.) (779) GACAGCCGTACCAGGTCTTCTTGATCTTCCTCGGTCTCACTCTGTTCTGCAACGCTGTCT
Consensus (781) GA AG CCTACCAGGTCTTCCTCGTCTTCCTCGGCCTTACA TCTTCTGCAACGCTGTCT
(841) 841 850 860 870 880 890 900
2013 all ORF's unedited (620) CCGCGCTTGGAAATAAGTGGCTCCCATGGTTGGACGTAAGTCAAAATGACTCCACTTCAA
EAA78258 (G. zeae hyp. prot.) (605) CTGCCCTCGGAAACAAATACCTTCCCTGG-------------------------------
XM_363459 (M. gris. hyp.pro.) (839) CGTCTCTTGGCAACAAGTGGCTGCCAATC-------------------------------
Consensus (841) C GC CTTGGAAACAAGTGGCT CCATGG
(901) 901 910 920 930 940 950 960
2013 all ORF's unedited (680) ACTCGTGAATTGACCGCGACAGACTTTTGCCATTTACTGGACTTTTGCCGGTGTTTTCGC
EAA78258 (G. zeae hyp. prot.) (634) --------ATTGAT--------ACTGCTGCTGTGTTCTGGACTTTTGCTGGTGTCATTGC
XM_363459 (M. gris. hyp.pro.) (868) ---C-----TGGAT--------ACTGCCGCCATTTTCTGGACCTTTGCTGGCGTCATTGC
Consensus (901) C ATTGAT ACTGCTGCCATTTTCTGGACTTTTGCTGGTGTCATTGC
(961) 961 970 980 990 1000 1010 1020
2013 all ORF's unedited (740) TATCGTCATTTGCGTTCTCGCGCTCGCCAAGGAGGGTAGACGATCTGCTAAATTCGTCTT
EAA78258 (G. zeae hyp. prot.) (678) CATTGTCGTTTGTGTTCTCGCCATGGCTAAGGAAGGCCGTCGCGATGCTGCCTACGTCTT
XM_363459 (M. gris. hyp.pro.) (912) GATTATGGTCACGGTCCTTGCGCTTGCCAAGGCCGGTCGCCGTGATGCTGCGTTTGTCTT
Consensus (961) ATTGTCGTTTG GTTCTCGCGCT GCCAAGGA GGTCG CG GATGCTGC TTCGTCTT

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                        (1021) 1021       1030        1040        1050        1060        1070      1080
2013 all ORF's unedited  (800) CACCGATTTCGAACCACTGGATGGATGGACTCCAGGATGGGCTTTCTTCGTTGGTCTCTT
EAA78258 (G. zeae hyp. prot.)  (738) TGGTCACTTTGAGGCCAACTCTGGATGGCCTAAGGGCTGGTCTTTCTGTGTCGGTCTGCT
XM_363459 (M. gris. hyp.pro.)  (972) TGGTCACTTTGAGACCAACTCTGGCTGGCCCGCCGGCTGGTCTTTCTGCGTTGGTCTGTT
               Consensus(1021) TGGTCACTTTGAG CCAACTCTGGATGGCCT C GGCTGGTCTTTCTGCGTTGGTCTGTT

                        (1081) 1081       1090        1100        1110        1120        1130      1140
2013 all ORF's unedited  (860) GCAAGCGGCATACGCAACCTCTTCCACGGGCATGATCATTTCGTAAGTTCATTGCGAAAG
EAA78258 (G. zeae hyp. prot.)  (798) CCACGCTGCCTACGCTACTTCTTCCACCGGCATGATCATCTC-------CAT--------
XM_363459 (M. gris. hyp.pro.)(1032) GCACGCCGGATATGCGACCTCGTCGACGGGAATGATCATCTC-------CAT--------
               Consensus(1081) GCACGC GCATACGC ACCTCTTCCACGGGCATGATCATCTC       CAT

                        (1141) 1141       1150        1160        1170        1180        1190      1200
2013 all ORF's unedited  (920) CACTTCAGAATCTGTCTAACAAGGATAGCATGTGCGAAGAAGTAGAAAACCCCAGCGTTC
EAA78258 (G. zeae hyp. prot.)  (843) -------------------------------GTGCGAGGAAGTACAGAACCCCTCGGTCC
XM_363459 (M. gris. hyp.pro.)(1077) -------------------------------GTGTGAGGAGGTTCGCATGCCCTCGACCC
               Consensus(1141)                                GTGCGAGGAAGTACA AACCCCTCGGTCC

                        (1201) 1201       1210        1220        1230        1240        1250      1260
2013 all ORF's unedited  (980) AAGTCCCCCGCGCCATGGTCGGAACCATCGTCCTTAACACCATCTGCGGACTTGTCTTCC
EAA78258 (G. zeae hyp. prot.)  (872) AGGTCCCCAAGGCCATGGTCGCCACCATCTTCATCAACACCTTCGCCGGTCTCCTCTTCA
XM_363459 (M. gris. hyp.pro.)(1106) AGGTCCCCAAGGCCATGGTCGCCACCGTTGTGCTCAACACCTTTGCCGGTCTGTTGTTCA
               Consensus(1201) AGGTCCCCAAGGCCATGGTCGCCACCATCGTCCTCAACACCTTCGCCGGTCT  TCTTCA

                        (1261) 1261       1270        1280        1290        1300        1310      1320
2013 all ORF's unedited(1040) TTGTCCCCCTCCTCTTCGTTCTCCCAGATCTCAAGATGCTCTATGGCATCGTCTCCGGCC
EAA78258 (G. zeae hyp. prot.)  (932) TCATCCCGTTGATGTTCGTCCTCCCCGATCTC------CAACAAGTCATCCTCTCTGCCC
XM_363459 (M. gris. hyp.pro.)(1166) TGATCCCCTTGGTCTTTGTCCTTCCCGACATCCAGTACCTGATCGGCCTCGCATCTGGTC
               Consensus(1261) T ATCCCCTTG TCTTCGTCCTCCCCGATCTC AG    CT   A GGCATCGTCTCTGGCC

                        (1321) 1321       1330        1340        1350        1360        1370      1380
2013 all ORF's unedited(1100) AACCAGTGCCCGTCATTATCGCGACAGCGGTCGGCAACAAAGCTGGTGCATTCGTCCTTC
EAA78258 (G. zeae hyp. prot.)  (986) AGCCAGTGCCCTTCATCATCAAGTCCGCTGTTGGCAGCTCCGGTGGTGCCTTTGGACTCC
XM_363459 (M. gris. hyp.pro.)(1226) AACCCGTTCCCGAGATCATCCTCGCCGCCGTCGGCGAACCCGGTGCTGCGATTGCGCTGC
               Consensus(1321) AACCAGTGCCCGTCATCATC   G CCGC GTCGGCAAC CCGGTGGTGC TTTG   CT C

                        (1381) 1381       1390        1400        1410        1420        1430      1440
2013 all ORF's unedited(1160) TCATACCTCTCTTGGTTCTTGCTATCTTCTGCGGTATTGGTTGCACGACCGCTGCGTCGC
EAA78258 (G. zeae hyp. prot.) (1046) TCTTCCCTCTCATCATCCTCGCCATCATCTGCGGTATTGGCTGCACAACTGCCACTTCTC
XM_363459 (M. gris. hyp.pro.)(1286) TCATGCCCCTGCTGGTCCTGGCCATCATTTGCGGTATCGGCTGCACGACCGCCGCCTCCC
               Consensus(1381) TCAT CCTCTC TGGTCCT GCCATCATCTGCGGTATTGGCTGCACGACCGCCGC TC C

                        (1441) 1441       1450        1460        1470        1480        1490      1500
2013 all ORF's unedited(1220) GCGCCACATGGGCTTTCTCCCGCGATGGAGCAATTCCTGGCTTCAAGTGGTGGAAGGTTG
EAA78258 (G. zeae hyp. prot.) (1106) GATGCACATGGGCTTTCGCTCGTGACGGTGCCATCCCTGGTGCTAAGTGGTGGTCCAAGG
XM_363459 (M. gris. hyp.pro.)(1346) GCTGCACTTGGGCTTTTGCTCGCGACGGTGCTATTCCTGGTGCACAGTGGTGGCGCGTCA
               Consensus(1441) GCTGCACATGGGCTTTCGCTCGCGACGGTGC ATTCCTGGTGC AAGTGGTGG   CGT G

                        (1501) 1501       1510        1520        1530        1540        1550      1560
2013 all ORF's unedited(1280) TCAACCACAGCCTTGACGTCCCGCTCAATGCTATGATGCTCAGTATGGCTGTTCAGATCC
EAA78258 (G. zeae hyp. prot.) (1166) TCAACACCTCGCTCGACGTTCCCCTCAACGCCATGATGCTCAGCATGGTTGTCCAGATCA
XM_363459 (M. gris. hyp.pro.)(1406) TCAACGAGAAGCTCGACGTGCCCTTGAACGCCATGATGCTCTCAATGGCCGTGCAGATCA
               Consensus(1501) TCAAC ACA GCTCGACGT CCCCTCAACGCCATGATGCTCAG ATGGCTGT CAGATCA

                        (1561) 1561       1570        1580        1590        1600        1610      1620
2013 all ORF's unedited(1340) TCGTCGGTTTCTTGTACTTCGGTTCCACGGCTGCTTTCAACGCTTTCTCCGGTGTTGGTG
EAA78258 (G. zeae hyp. prot.) (1226) TCCTCGGTGTCATCTACTTTGGTTCATCCGCCGCCTTCAACGCCTTCTCCGGTGTTGGTG
XM_363459 (M. gris. hyp.pro.)(1466) TCCTCGGCCTCATCTACTTTGGCTCGTCGGCTGCATTCAACGCCTTCTCCGGCGTCGGCG
               Consensus(1561) TCCTCGGT TCATCTACTTTGGTTC TCGGCTGC TTCAACGCCTTCTCCGGTGTTGGTG
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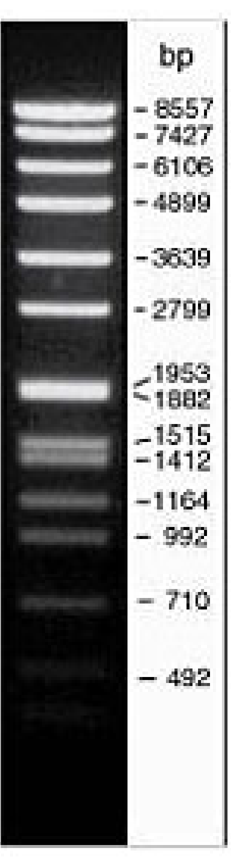
(1621) 1621 1630 1640 1650 1660 1670 1680
2013 all ORF's unedited(1400) TCATTTGCTTAACAGTCAGCTACGCCGTTCCTGTCGCAGTCTCCTTGATCGGTGGCCGTT
EAA78258 (G. zeae hyp. prot.) (1286) TCATTTGCTTGACTGCCTCTTACGCTACTCCCATTGCCATCAGTCTCGCCACTGGCCGTA
XM_363459 (M. gris. hyp.pro.)(1526) TCATCTGCCTCACGGCCTCGTACGCCACCCCGATCGCCATCAACCTGTTCAAGGGTCGCA
Consensus(1621) TCATTTGCTT AC GCCTC TACGCCACTCC ATCGCCATCA CCTG TCA TGGCCGTA
(1681) 1681 1690 1700 1710 1720 1730 1740
2013 all ORF's unedited(1460) CTCATCTAAAGTATGGAAAATTCGACAAGGGAAAGCTCGGTCTGTTCTGCAACATTGTTT
EAA78258 (G. zeae hyp. prot.) (1346) AGCAGGTCAAGACTGGAAAGTTCTACCTTGGCAAATTTGGCGCTGTCGCCAACGTCATT-
XM_363459 (M. gris. hyp.pro.)(1586) AGGCTACCGCCAACGCCAAGTTCCAGCTCGGTCGCATGGGAGTCTTTTGCAACATTGTC-
Consensus(1681) AGCAT TCAAGAATGGAAAGTTC ACCT GG AA T GG GT TTCTGCAACATTGTT
(1741) 1741 1750 1760 1770 1780 1790 1800
2013 all ORF's unedited(1520) CCCTCGGTACGCTATATCCTCTCTACTTGAGTCCTTAGTTTTTGTTTTGCTGACCTTACC
EAA78258 (G. zeae hyp. prot.) (1405) ----------GCTAT----------C----------------------------------
XM_363459 (M. gris. hyp.pro.)(1645) ----------GCCCT----------C----------------------------------
Consensus(1741) GCTAT C
(1801) 1801 1810 1820 1830 1840 1850 1860
2013 all ORF's unedited(1580) CAGCCTGGTCTGTTCTCGTAGTTCCCCTCTTCTGCATGCCTTCCTACCTTCCTGTCGCAG
EAA78258 (G. zeae hyp. prot.) (1411) --GCCTGGTCTCTTCTCGCCATGCCTCTCTTCTGCATGCCCTCCATGATCCCTGTTACCC
XM_363459 (M. gris. hyp.pro.)(1651) --GCCTGGTCTGCGCTCGCCATGCCGCTCTTTTGCATGCCGAGCTACCTGCCCGTCACGC
Consensus(1801) GCCTGGTCTGTTCTCGCCATGCC CTCTTCTGCATGCC TCCTACCT CCTGTCAC C
(1861) 1861 1870 1880 1890 1900 1910 1920
2013 all ORF's unedited(1640) CTGAGACGATGAACTACGCCTCGGTCGTCTTCGTCGCCTTCTTCCTCGTCGCTGCAGGGT
EAA78258 (G. zeae hyp. prot.) (1469) CCGAAACCGTCAACTACGCCCCCGTCGTCTTCGTCTTCGCATGCCTTGTTTCTGGAATTT
XM_363459 (M. gris. hyp.pro.)(1709) CCGAGACGGTCAACTACGCGCCCGTGGTCTTTGTCGCAGCCACCATCGTTTCTGGTGTGT
Consensus(1861) CCGAGACGGTCAACTACGCCCCCGTCGTCTTCGTCGCCGCCT CCTCGTTTCTGGAGTGT
(1921) 1921 1930 1940 1950 1960 1970 1980
2013 all ORF's unedited(1700) GGTACTTCGTCTGGGGCAAGAAGAACTACGCTGGCCCACCTGTCCAGGAAGACGCCGCTA
EAA78258 (G. zeae hyp. prot.) (1529) GGTACTGGGCCTGGGGTCACAAGAACTACGCTGGTCCCCCACCAACGAGGATTAG----
XM_363459 (M. gris. hyp.pro.)(1769) GGTACATTGTGTGGGGCAGGGAGAACTACGCCGGCCCTCCAGTCCAGGAGGAGTACAACT
Consensus(1921) GGTACTT GTCTGGGGCAAGAAGAACTACGCTGGCCC CC GTCCAGGAGGA TAC
(1981) 1981 1990 2000 2010 2020 2036
2013 all ORF's unedited(1760) TTGAGCGTCGCAGGTCTGAAATTGGCGCGCATCCTCATGAGCTTCCAAGTGAATGA
EAA78258 (G. zeae hyp. prot.) (1585) --
XM_363459 (M. gris. hyp.pro.)(1829) AG--
Consensus(1981)

Appendix D: Size Marker – SPP-1 Phage DNA/*Eco*RI

SPP-1 Phage DNA digested with the *Eco*RI restriction enzyme (Geneworks Pty Ltd., Hindmarsh, SA, Australia)



Appendix E: DNA Sequences

YB4.20 DNA Sequence

1 ctgtttcctc ctcaaccgtg ctgtggttac tgttcagttg cgttacaagg tggactagtg
61 cctccttgaa tggacgcgta tcatgaagct ttaagttgaa agaaacaaat cgcgaatttc
121 tataatcaag aataatattc cttcgtctta ctaacatcag gcggtcccca aatctttgtc
181 gtcatcccca ttccctcggc tctctcaata ctcgtaatac tatcactcgc ctcgtgcttg
241 aaactcttct tcgtatcctg tttcaatggg acatgatcat cctgtgcttc tgcaggaatc
301 ctctcatccc agctcttcgt caaagctgaa gcgtccacac tcaaagcctc actactcttc
361 ctctgctcga caacaacatg aacctctcgt ttcgtgttga acgttatatc accagttcca
421 tgagcggatg agccggatct ggctgagtcg gcccttttcc agttcacatc ttgacttgtc
481 ccgaatgtct ttgtgagttg agtagcgatg agattttggt ggttgcccat ggagtctatg
541 ttattgttcg tatctgaagc tatccgagcg atcatgtcgg ccattgacat ttcgatgttg
601 agttttacca tgtaggcgac ggggtggaat tgcatgtaac tattcgaatg atggtgatta
661 gctatagagc ctgtttgatc gaagcatgtg gaacttacac aaagctattg ttcaagctca
721 tcatcgataa gatcatacaa tccattgcaa gagaggataa cgacaatgcc ctgggttgaa
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841 gaatgaagta aaagttcaat gctccatccg caatcaagta catgcctttc tcacatcggt
901 cccagacttc attgatccgg atgtaccgct ctgagatttg gagacgagct gggatcccta
961 aaaacgtctg acattagttt ttggcgtgtg atgcggatat cattgtttga gaacttacat
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1081 caaggcttta acctgatccg gaaggagtaa tgccactcgg ttgatgataa tctggagcaa
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1321 ccagcagata acagagaaga tcaagcagac gaggatttcc aaccatatca taataatgta
1381 cggcgaatgg actctagaaa acccatatcg cttggcaacc tctgtggtct gcttgaaggc
1441 agtccctatc accaagcata ttaatacacg tcctccattt acgatattgg aagtttgttg
1501 cgatcgactt acatgtggtg agaaaaccaa aaccaagagt gaacccccaa gcaatagatg
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1981 cgaaacttcg tccacgaggt aacgaggagg aaggccaatg ggagcctccg agctcaaatt
2041 acattatatc agcatgccaa tctggggtgt caaagaccgt attcgagtgt tagatcagaa
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2701 caagtaatgg gcttgttacc aaagtcagaa caccaattgg atgtaaaagc cacgaagtgc
2761 actactcgtt tagcagtttg acggcggatc cgaggatccc taaacgttgt ttcatgccag
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4981 gtaagaagga ggtttttgtc aattttctgc gtgctcgctt gtttggtggg cgaataatat
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YB4.44 DNA Sequence

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181 ttttctttac aaatctagat tcgagacaca ttgttttgat tcttgcgact acagaatcct
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301 ctttcaactt gtatgttgaa caccctcgca cccaccgaaa cagatcccat gtaatcatga
361 tcatattaag aaagaatcct acggctttat caatacataa gtctcctact gaacatatct
421 ccactcgaca tactctccca tcatatcatc cttcttcaag cattgaagct gctcccttcc
481 taatcccaaa aaaacctcca agcctccact tttctcctcc ccagatccct ctataatcct
541 tggaaaaaca acgaaaatcc catcactgat cttattcctc cacctcaccc tctcatatct
601 accgctaaga gtcttgcccc aatcaaggga gtaatagctc tggctagcct aagacgtaca
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781 ccggagagta gcagcgatac ctgagaggtt gacagtggcg accaatgtgt ctgtagtttg
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901 tcgactacgc ctattgacga ccatgttgaa catcgagtaa gggattcccc ctatccctgt
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4681 tcgactgttt aatctgcggg atgactgggt tccgatctgc catcaatcat catcataata
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YB7.395 DNA Sequence

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YH4.5 DNA Sequence

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LH2013 DNA Sequence

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