CHARACTERIZATION OF Yr15 RESISTANCE TO STRIPE RUST OF WHEAT

By

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<tbody>
<tr>
<td>A</td>
<td>absorbance</td>
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<tr>
<td>A.D.</td>
<td>Anno Domini</td>
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<td>AFLP</td>
<td>amplified fragment length polymorphism</td>
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<td>AP</td>
<td>abortive penetration</td>
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<td>ASSV</td>
<td>aborted substomatal vesicle</td>
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<tr>
<td>ASSVN</td>
<td>aborted substomatal vesicle with necrosis</td>
</tr>
<tr>
<td>B.C.</td>
<td>Before Christ</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>cDNA</td>
<td>chromosomal deoxyribonucleic acid</td>
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<tr>
<td>CIMMYT</td>
<td>International Maize and Wheat Improvement Centre</td>
</tr>
<tr>
<td>ca</td>
<td>circa (about)</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpi</td>
<td>days post-inoculation</td>
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<tr>
<td>e.g.</td>
<td>exempli gratia (for example)</td>
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<tr>
<td>et al.</td>
<td>et alii (and others)</td>
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<td>Fig.</td>
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<tr>
<td>G</td>
<td>germ tube</td>
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<td>g</td>
<td>gram</td>
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<td>f. sp.</td>
<td>forma specialis</td>
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<td>H</td>
<td>haustorium</td>
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<td>HMC</td>
<td>haustorium mother cell</td>
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<td>HN</td>
<td>haustorium neck</td>
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<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>hpi</td>
<td>hours post-inoculation</td>
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<tr>
<td>i.e.</td>
<td>id est (that is)</td>
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<tr>
<td>IH</td>
<td>infection hypha</td>
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<td>Inc.</td>
<td>incorporated</td>
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<td>Abbreviation</td>
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</tr>
<tr>
<td>IWF</td>
<td>intercellular washing fluid</td>
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<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>kV</td>
<td>kilovolt</td>
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<tr>
<td>Lr</td>
<td>leaf rust resistance gene</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>m</td>
<td>meter</td>
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<tr>
<td>MAS</td>
<td>marker-assisted selection</td>
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<td>mg</td>
<td>milligram</td>
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<td>min</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>m/v</td>
<td>mass/volume</td>
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<tr>
<td>nm</td>
<td>nanometre</td>
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<tr>
<td>no.</td>
<td>number</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PIH</td>
<td>primary infection hypha</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethyl-sulfonylfluorid</td>
</tr>
<tr>
<td>PR</td>
<td>pathogenesis-related</td>
</tr>
<tr>
<td>QTL</td>
<td>quantitative trait loci</td>
</tr>
<tr>
<td>RAPD</td>
<td>random amplified polymorphic DNA</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>S</td>
<td>stoma</td>
</tr>
<tr>
<td>SAR</td>
<td>systemic acquired resistance</td>
</tr>
<tr>
<td>SCARs</td>
<td>sequence characterised amplified regions</td>
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<tr>
<td>SEM</td>
<td>scanning electron microscope</td>
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<tr>
<td>SOD</td>
<td>superoxide dismutases</td>
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<tr>
<td>Sr</td>
<td>stem rust resistance gene</td>
</tr>
<tr>
<td>SSV</td>
<td>substomatal vesicle</td>
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<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>SSVI</td>
<td>substomatal vesicle initial</td>
</tr>
<tr>
<td>STSs</td>
<td>sequence tagged sites</td>
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<tr>
<td>TMV</td>
<td>tobacco mosaic virus</td>
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<tr>
<td>t/ha</td>
<td>ton per hectare</td>
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<tr>
<td>U</td>
<td>urediniospore</td>
</tr>
<tr>
<td>UOFS</td>
<td>University of the Orange Free State</td>
</tr>
<tr>
<td>U.S.A</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet light</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
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<tr>
<td>Yr</td>
<td>stripe rust resistance gene</td>
</tr>
<tr>
<td>%</td>
<td>percentage</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celcius</td>
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<td>μ</td>
<td>micro</td>
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<tr>
<td>μg</td>
<td>microgram</td>
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<tr>
<td>μmol</td>
<td>micromole</td>
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Chapter 1

GENERAL INTRODUCTION

Cereal crops account for the staple diet of 90% of the world’s population. Collectively, these crops provide two-thirds of the carbohydrate intake and a large proportion of the protein requirements of humankind. Food production and food security play a significant role in the sustainability of communities. The pathogens that cause disease and yield losses in these crops hold serious economic implications for both the producers and the countries that rely on them for staple foods (Moore et al., 1993).

Cereal crops are particularly important in South Africa which is a developing country with an ever increasing population. Wheat has been grown in South Africa since the middle of the 17th century. One of the first undertakings of Jan van Riebeeck, after his arrival at the Cape in 1652, was to sow wheat on the site of present day Cape Town. During the 18th century wheat production expanded gradually as the early pioneers settled in new areas. It is recorded that in 1752 the crop was being grown in well-known wheat areas such as the Swartland and Overberg (Du Plessis, 1933). According to McIntosh et al. (1995) it is generally accepted that any stress factor negatively influencing wheat production would thus be detrimental to the economy of a country.

Stripe (yellow) rust, caused by *Puccinia striiformis* Westend f. sp. *tritici*, is a major disease of bread wheat, *Triticum aestivum* L. The disease is confined to cooler climates and is most prevalent in north-western Europe and the Mediterranean region, as well as certain areas of the U.S.A. According to Danial (1994), stripe rust has recently become a major threat to wheat production in Kenya and other wheat growing areas in East Africa. Factors which have contributed to this are:
1) breeders concentrated mainly on developing cultivars resistant to stem rust without realising the importance of stripe rust

2) national programmes in East Africa are mainly dependent on advanced International Maize and Wheat Improvement Centre (CIMMYT) wheat lines, which are released after a short period of testing. Soon after the release of such introduced lines the wheat cultivars often become susceptible due to the appearance of new races of the stripe rust pathogen. The cultivar Paa released in 1982 became susceptible in 1984, providing an example of what is typically known as the boom and bust cycle (Danial, 1994).

Stripe rust can be as damaging as stem rust. However, the lower temperature requirement for optimum development limits stripe rust as a major disease in many wheat growing areas of the world. The minimum, optimum and maximum temperatures for spore germination are 0°C, 11°C and 23°C, respectively. Stripe rust is principally a disease of wheat during the winter or early spring or at high elevations. In Europe a forma specialis of \textit{P. striiformis} has evolved that is commonly found on barley and seldom on any but the most susceptible wheats. \textit{Puccinia striiformis} f. sp. \textit{hordei} was introduced to South America where it spread across the continent as well as northwards into North America (Roelfs \textit{et al.}, 1992).

The first appearance of stripe rust in South Africa was recorded in 1996 (Pretorius \textit{et al.}, 1997). After being introduced to the Western Cape it spread rapidly throughout the wheat producing areas of the province, causing widespread and severe losses on susceptible cultivars. In early 1997 the disease was detected in the Western Free State from where it spread throughout the rest of the country. In 1998 a new pathotype, 6E22, evolved on winter wheat grown in the eastern
Free State (Boshoff and Pretorius, 1999). Although stripe rust occurs in all wheat growing areas, epidemics have not been encountered in warm and dry environments (Kloppers et al., 1997).

Infection with stripe rust may occur at any stage of plant development. Germination of urediniospores on the leaf occurs following contact with water. The stripe rust fungus is characterised by its systemic growth in the leaf, typically producing uredinia in narrow, linear stripes mainly on leaves and spikelets. When the heads are infected, pustules appear on the inner surface of glumes and lemmas, occasionally invading the developing kernels (Danial, 1994).

Effective chemical control of stripe rust is available, but is expensive and not economically viable in developing countries or in areas where lower yields are obtained. The continued use of fungicides may become an environmental hazard, as well as ineffective due to reduced sensitivity in the pathogen.

Resistance to stripe rust is often of the race-specific, major-gene type. A series of such resistance genes have been identified. McIntosh et al. (1995) reported 18 officially designated Yr (yellow rust) genes, as well as additional sources of resistance to this disease. Major-gene resistance is characterised by a low infection type and has become ineffective in many areas due to genetic adaptations in the pathogen (Stubbs, 1985). However, several cultivars have remained resistant after prolonged and widespread cultivation. Cultivars with durable resistance to stripe rust provide an acceptable control strategy as they reduce environmental pollution, avoid tolerance or resistance in the pathogen, and prevent the boom and bust cycle introduced with nondurable resistance (Danial, 1994). Such cultivars have been reported from many parts of the world (Line, 1978; Johnson, 1988; Van Dijk et al., 1988). In many of these, resistance was incidental and
did not result from breeding programmes directed at durability (Danial, 1994).

The development of stripe rust-resistant wheat cultivars has become a high priority in the South African wheat industry. This effort is focused on the identification of sources of genetic resistance and the incorporation of those resistance genes into locally adapted wheat cultivars.

The objective of this study was to evaluate the \textit{Yr15} gene as a potential source of stripe rust control in South Africa. Studies included histopathology, pathogenesis-related proteins, inheritance and molecular markers.
Chapter 2
LITERATURE REVIEW

2.1 THE HISTORY OF WHEAT RUSTS

It is well documented that the occurrence of rust diseases in cultivated cereals has significantly influenced human civilisation (McIntosh et al., 1995). Aristotle (384-322 B.C.) first wrote of rust being produced by "warm vapours" and mentioned the devastation caused in epidemic years. Excavations in Israel have revealed urediniospores of stem rust dating to ca 1300 BC. Rituals involving the Roman god, Robigus, emphasise the fact that rust was a serious disease in Europe during that period.

Although the biology was not understood, an observed relationship between rusted cereals and diseased barberry was appreciated from early times. Measures to control barberry eventually culminated in the expensive and often controversial barberry eradication program in the U.S.A. in the early part of this century (McIntosh et al., 1995).

The Italians Fontana and Tozzetti provided the first detailed reports of wheat stem rust in 1767 (Roelfs et al., 1992). It was not until well into the 19th century that a distinction among rust diseases was made. Eriksson in Sweden defined *formae speciales* (f. sp.) to describe "special forms" of the wheat rust pathogens that showed specialisation on different host species (McIntosh et al., 1995).

According to Danial (1994) stripe rust was first described by Gadd in 1777 and as a pathogen of rye by Bjerkander in 1874. Schmidt described stripe rust as *Uredo glumarum* in 1827. Westendorp described stripe rust collected from rye as *Puccinia straminis*. In 1894 Eriksson and Henning showed that this rust differed from other rusts at
species level and named it *Puccinia glumarum* (Danial, 1994). This designation remained valid until Hylander et al., followed by Cummins and Stevenson (1956), introduced the name *Puccinia striiformis* Westend (Manners, 1960). The common names of yellow rust and stripe rust were given by Humphrey et al. in 1924 and by Eriksson and Henning in 1894, respectively (Danial, 1994).

The extensive effort directed at cereal rusts and their control since the 1880s, both in terms of science and practical efforts to prevent losses, led Large to observe that “the greatest single undertaking in the history of plant pathology was to be the attack on rust in cereals”. This remains a focus of plant pathology and breeding (McIntosh et al., 1995).

2.2 RUST FUNGI AND THE DISEASES THEY CAUSE

The plant rusts, caused by Basidiomycetes in the order Uredinales, are among the most destructive plant diseases. There are approximately 4000 species of rust fungi, several of which have caused famines and ruined the economy of large areas, including entire countries (Agrios, 1988). These fungi are most notorious for their destructiveness on grain crops, especially wheat, oats, and barley, but they also attack vegetables such as asparagus, field crops such as groundnuts, cotton and soybean, ornamentals such as carnation and snapdragon, and have caused extensive losses on trees such as pine, apple, and coffee (Agrios, 1988; McIntosh et al., 1995).

The rust fungi are obligate parasites expressing both simple and complex life cycles. These fungi typically produce five distinct reproductive stages, each with a different spore form, some of which are parasitic on one host whereas the others are parasitic on an alternate host (Barnes, 1979).
The rust fungi attack mostly leaves and stems and occasionally floral parts and fruits. Infections usually appear as rusty, orange, yellow or even white-coloured lesions that typically disrupt the epidermis, the formation of swellings, and even galls. Most rust infection sites are localised, but some may spread systemically in the plant (Bilgrami and Dube, 1976).

Roelfs et al. (1992) described the three rust diseases that occur on wheat i.e. brown, black and stripe rust. The causal organisms of these rusts are *Puccinia recondita* f. sp. *tritici*, *P. graminis* f. sp. *tritici* and *P. striiformis* f. sp. *tritici* respectively. Of these, the most common is leaf or brown rust. Leaf rust occurs primarily on the leaf blades, although leaf sheaths can also be infected. *Puccinia recondita* f. sp. *tritici* frequently lacks abundant teliospore production at the end of the season, resulting in brown rather than the black lesions of stem rust. Leaf rust teliospores usually appear from telia on the lower leaf surfaces and remain covered by the epidermis. The disease develops at temperatures between 10 and 30°C. Leaf rust occurs at varying levels of severity wherever wheat is grown and losses are primarily attributed to a reduction in grain number and mass. In susceptible genotypes, florets, tillers, and plants can be killed by early (pre-heading) epidemics. Losses due to leaf rust are usually small (<10%), but may be severe under favourable conditions (>30%).

Based on the abundant production of dark-coloured teliospores, stem rust is also known as black rust. This disease is favoured by humid conditions and warmer temperatures ranging between 15 and 35°C. It is considered the most devastating of the wheat rusts and may cause losses of 50-100% in highly susceptible cultivars.

Stripe rust derives its name from the characteristic uredinial stripes producing yellow-coloured urediniospores in uredinia. Because this
disease often occurs during early growth stages, stunted and weakened plants are common during epidemics. Losses can be severe (50%) due to a reduced tiller number and shrivelled grain and, in extreme situations, the entire crop may be destroyed (Roelfs et al., 1992).

2.3 INFECTION OF WHEAT BY RUST FUNGI

The infection of a cereal host by a rust fungus and its subsequent development consist of several distinct stages: germination of urediniospores and formation of appressoria, substomatal vesicles, primary and secondary infection hyphae as well as their growth, haustorium mother cells, haustoria, uredinia and urediniospore production (Lee and Shaner, 1984).

The infection process can be described during the prepenetration (epidermal) and postpenetration (sub epidermal) stages. According to Roelfs et al. (1992), Broers and Jacobs further divided the prepenetration stage into germination, germ tube growth and orientation, and appressorium formation. The urediniospores of *P. striiformis* germinate in the dark at high humidity. Ideal temperature conditions range from 9 to 13°C (Roelfs et al., 1992). Allen et al. (1991) documented that once germination has taken place, the germtube, which appears as a protrusion from the spore, elongates and grows towards the stomata. Their study also noted that chemical and physical stimuli affect the growth and orientation of the germtube (Jacobs, 1996). *Puccinia striiformis* differs from the other wheat rusts by not forming clearly differentiated appressoria (Roelfs et al., 1992).

Postpenetration behaviour can also be categorized in various phases of fungal development. Once an appressorium has been formed, an infection peg is initiated (Littlefield and Heath, 1979). According to Staples and Macko (1980) this infection peg then proceeds to
penetrate the stomatal cavity and swells to form a substomatal vesicle. They also noted that the substomatal vesicle gives rise to the development of single or multiple infection hyphae and, that contact between the primary hyphae and a mesophyll cell forms a septum that lays the foundation for a haustorium mother cell (Jacobs, 1996). A peg develops from the mother cell and penetrates the mesophyll cell to form an intracellular haustorium. Secondary infection hyphae undergo differentiation, giving rise to additional haustorium mother cells and haustoria, eventually resulting in extensive colonisation of host tissue (Heath, 1977).

Initially rust fungi develop in a similar way in compatible and incompatible hosts. Several mechanisms of resistance have been demonstrated in histological studies of the interaction between plants and rust fungi. Heath (1981) distinguished between the broad mechanisms of pre- and posthaustorial resistance. Prehaustorial resistance refers generally to an arrestation of fungal development prior to the formation of the first haustorium (Heath, 1977), whereas posthaustorial resistance is characterised by the confinement of the pathogen after formation of at least one haustorium (Jacobs, 1996). An active defense system occurring in most higher plants in response to pathogens is the hypersensitive response. This is characterised by rapid cell death at the infection site (Keen, 1990).

More specifically, Heath (1981) mentioned that plants possess a number of defense mechanisms which may include:

1) inhibition of germination
2) barriers to prevent penetration of plants
3) inhibition of infection structure formation
4) inhibition of haustorium formation.
Earlier studies by Heath (1974) have suggested that there are at least three mechanisms by which formation of the haustorium could be prevented:

1) osmophilic material is deposited on and within the adjacent non-host cell wall
2) haustorium mother cells lose contact with non-host cells relatively easy
3) fungal death occurs prior to haustorium initiation.

2.4 EPIDEMIOLOGY OF STRIPE RUST

*Puccinia striiformis* f. sp. *tritici* has the lowest temperature requirements of the three rust pathogens of wheat. Although the optimum is 11°C, infection may occur between a minimum and maximum of 0 and 23°C, respectively (Roelfs *et al.*, 1992).

According to Roelfs *et al.* (1992) *P. striiformis* f. sp. *tritici* oversummers on wheat in Europe. The amount of oversummering inoculum depends on the availability of volunteer wheat which, in turn, is dependent on rainfall in the off-season. In north-western Europe, where extremely low temperatures kill sporulating lesions, overwintering is limited to mycelia in living leaf tissue. Snow may insulate sporulating lesions, thus supporting survival of the fungus. During winter, the latent period for this fungus can be as long as 118 days and may even extend to 150 days under snow cover (Zadoks, 1961).

According to Saari and Prescott (1985), in areas near the equator, stripe rust cycles endemically from lower to higher altitudes following crop development. In more northern latitudes, the disease moves over greater distances from mountainous areas to plains.

The susceptibility of stripe rust urediniospores to ultraviolet light restricts the long distance transport of viable propagules. Maddison
and Manners (1972) reported that these urediniospores are three times more sensitive to ultraviolet light than those of *P. graminis*. However, Zadoks (1961) reported that stripe rust was transported by wind in a viable state for more than 800 km. The introduction of wheat stripe rust into Australia and barley stripe rust into Colombia was most likely assisted by humans through jet travel (O'Brien *et al*., 1980; Dubin and Stubbs, 1986). It is generally accepted that the spread of stripe rust from Australia to New Zealand, over a distance of 2000 km, was probably through airborne inoculum (Beresford, 1982). The mode of introduction of this disease into South Africa is not known. The identity of the exotic introduction, viz. pathotype 6E16, suggests that it most likely originated on the African continent (Z.A. Pretorius, personal communication, 1999).

In many areas of the world volunteer wheat appears to play a significant role as an overseasoning or primary source of inoculum. Evidence implicating non-cereal grasses as inoculum sources also exists (Hendrix *et al*., 1965; Tollenaar and Houston, 1967). According to Roelfs *et al*. (1992) stripe rust epidemics in the Netherlands can be generated by a single uredinium per hectare, provided the spring season is favourable for disease development.

**2.5 HOST-PATHOGEN INTERACTIONS**

Roelfs *et al*. (1992) divided host-pathogen interactions into specific and non-specific associations.

**2.5.1 Specific interaction**

Specific interactions occur when different pathogen isolates produce different disease reactions on the same host in the same environment. According to Flor (1956) the gene-for-gene theory is based on specific interactions. Three assumptions with regard to this theory have been made, although some exceptions occur:
1) specific resistance is due to dominant genes in the host
2) dominance is complete, and
3) avirulence is dominant.

A cultivar never loses its genetic resistance to an isolate. However, various factors may result in unexpressed or ineffective resistance. Furthermore, a cultivar may be resistant to certain isolates but susceptible to others, and an isolate may be virulent on some cultivars but avirulent on others. In cereal rust terminology the disease phenotype is called the infection type. These infection types are the product of an interaction between the environment, host and pathogen. Seedling infection types are generally scored on numeric scales. Higher ratings, i.e. either a 3 or 4 on the commonly used 0-4 scale, and a 7, 8 or 9 on the 0-9 scale, are considered to indicate susceptibility. The extent of incompatibility between a pathogen and host is reflected by lower infection types (Roelfs et al., 1992).

Incompatibility can be expressed either as an immune response if resistance onset is early, or it may be expressed as a reduction in sporulation at later stages. When more than one specific resistance gene is present, the most effective gene will determine the resistant phenotype (Roelfs et al., 1992).

German and Kolmer (1990) indicated that, in combinations, specific and non-specific resistance genes may have an additive or complementary effect. Evidence has also been provided that the host genome affects the way in which this interaction is expressed (Roelfs et al., 1992).

2.5.2 Non-specific interaction
Non-specific interactions occur when all isolates produce a similar response on a given host genotype. Based on the short lifespan of
specific resistance, it is generally accepted that plant breeding should focus on non-specific resistance to diseases. However, it is difficult, if not impossible, to prove non-specificity as every member of the pathogen population would have to be tested. Resistance classified as adult-plant, horizontal, slow-rusting, partial, minor gene, etc. are often placed in this group. (Roelfs et al., 1992).

2.6 PLANT DEFENSE MECHANISMS
In general, plants defend themselves against pathogens by a combination of systems (Agrios, 1988; Cramer et al., 1993):

1) structural characteristics act as physical barriers (passive resistance) which inhibit the pathogen from gaining entrance and spreading through the plant; and

2) induced biochemical reactions (active resistance) occur in plant cells and tissues and which produce substances that are either toxic or inhibiting to pathogen growth.

When referring to plant-pathogen interactions one should distinguish between plant immunity and plant resistance. Immunity of a plant towards a particular pathogen could be interpreted as the inability of that pathogen to enter the host. In some instances, pathogens penetrate their host only to be immediately prevented from further colonisation by the death (hypersensitivity) of the first living cells they penetrate. The resistant response differs between plant-pathogen interactions. These vary from high levels of resistance, where no visible symptoms are manifested, to low levels of resistance, where the host completely succumbs to disease (Dickinson and Lucas, 1977; Parry, 1990).

Disease resistance that is genetically controlled by the presence of one (monogenic) or more (oligogenic or polygenic) genes, is known as true
resistance. Here, the host and pathogen are incompatible, either because of a lack of recognition between them, or because the host plant can successfully defend itself against the pathogen. Two kinds of true resistance, i.e. vertical and horizontal (as introduced by Vanderplank in 1963), have been recognised (Agrios 1988; Parry, 1990).

2.6.1 Vertical resistance
Vertical resistance is characterised by a specific interaction between the host and its parasite and was defined as being effective against some races but ineffective against others (Vanderplank, 1963). It has, therefore, been named race-specific resistance and is relatively simply inherited (Dyck and Kerber, 1985). A host plant containing vertical resistance usually responds with a hypersensitive reaction, preventing the pathogen to become established and multiply. Vertical resistance inhibits the development of epidemics by limiting the initial inoculum (Vanderplank, 1963; Agrios, 1988; Parry, 1990).

2.6.2 Horizontal resistance
Horizontal resistance is analogous to the non-specific host-parasite interaction discussed above. In this case plants have a certain level of resistance effective against all races of a respective pathogen. Such resistance is sometimes called non-specific or quantitative, but most commonly referred to as horizontal resistance (Vanderplank, 1963). Horizontal resistance is controlled by many genes, therefore "polygenic" or "multi-gene resistance" are often used as synonyms. Each of these genes alone may be ineffective against the pathogen. Generally, horizontal resistance does not protect plants from becoming infected, it slows down the development of individual infection sites resulting in a slower rate of disease progress in the field (Vanderplank, 1963; Agrios, 1988; Parry, 1990).
2.6.3 Adult-plant, seedling and partial resistance
Resistance that is first visible in older plants is called adult-, mature-plant or post-seedling resistance (Dyck and Kerber, 1985). According to Denissen (1993), Zadoks characterised adult-plant resistance as a resistance which is not expressed in the seedling stage but develops with advancing plant age. Conversely, seedling resistance is easily detected in primary leaves and subsequently through all developmental stages of growth. Robinson (1976) stated that adult-plant resistance is of the horizontal type. However, race-specificity has been found for several of the adult-plant genes for resistance to cereal rusts. Although certain assumptions have been made, these terms have no genetic basis and merely describes the onset of resistance in terms of plant growth stage.

Certain forms of resistance (e.g. partial resistance, slow rusting) influence the rate of epidemic development by retarding disease progress (Vanderplank, 1963). Rate-reducing resistance was described by Parlevliet (1978) as resistance “characterised by a reduced rate of epidemic development in spite of a susceptible infection type”. This resistance has also been called slow rusting and several reports describing its occurrence in wheat exist (Caldwell et al., 1970; Ohm and Shaner, 1976; Statler et al., 1977; Gavinlertvatana and Wilcoxson, 1978; Kuhn et al., 1978; Shaner et al., 1978; Milus and Line, 1980; Shaner and Finney, 1980).

2.6.4 Durable resistance
The controversies associated with terminology pertaining to the concept of non-specific (horizontal) resistance led Johnson (1979; 1981; 1983) to propose the term durable resistance. Johnson (1981) defined durable resistance as “resistance that has remained effective in a cultivar during its widespread cultivation for a long sequence of generations or period of time, in an environment favourable to a
disease or pest". The advantage of this term is that it describes what has actually been observed without implying any genetic basis. Knott (1989) proposed two reasons for resistance being durable. Firstly, the pathogen is unable to develop virulence or the virulent races are not competitive with the avirulent races. Secondly, the virulent races do not, for some reason, come into contact with the resistant host.

2.6.5 Biochemical mechanisms of resistance
According to Bowles (1990) the extracellular matrix in plants is continuous with a system of intercellular air space. Collectively, this space is known as the apoplast which plays a central role in the defense strategy against stress factors. Pathogens that colonise an intact plant must enter via the apoplast. Furthermore, many pathogens use the apoplast as an internal transport system whereas others remain there (Bowles, 1990).

The apoplast is now recognised as the site at which signals originate to elicit defense responses, and where many defense-related products accumulate (Bowles, 1990). Substances dissolved in the free water of the apoplast, or those weakly bound to the cell wall, may be obtained by preparing intercellular washing fluids (IWF) (Rohringer et al., 1983).

Following pathogenic attack, some plants activate the expression of defense-related proteins (Bowles, 1990; Van Loon, 1997). These proteins are divided into different classes based on their function. They include structural proteins in the extracellular matrix, enzyme inhibitors, such as amylase and proteinase inhibitors, toxic proteins, such as lectins and thionins, and pathogenesis-related (PR) proteins (Bowles, 1990).

PR proteins were first identified in tobacco (Nicotiana tabacum) reacting hypersensitively to the tobacco mosaic virus (TMV)
(Gianinazzi et al., 1970; Van Loon and Van Kammen, 1970). These proteins do not only accumulate locally in the infected tissues, but are also involved in the development of systemic acquired resistance (SAR) (Ryals et al., 1996; Sticher et al., 1997). PR proteins may also be induced by different abiotic stresses, such as cutting, injury, high osmotic pressure (Ohashi and Ohshima, 1992), heavy metals (Curz-Ortega and Ownby, 1993), freezing (Honn et al., 1995) and several chemicals (Van Loon, 1985). The increased expression and accumulation of PR proteins during different stress conditions suggest that they are stress-related proteins in a general sense (Van Loon, 1997).

2.6.5.1 Classification of PR protein families

The common characteristics of PR proteins are:

1) selective extractibility at low pH
2) high resistance to proteases
3) localization in the vacuole and/or the apoplast and
4) low molecular weight (8-50 kDa) (Stintzi et al., 1993).

According to a recent classification proposed by Van Loon and Van Strien (1999), the PR proteins are divided into 14 families. Group 1 corresponds to PR-1 tobacco proteins with unknown function. The PR-2 family consists of endo-β-1,3-glucanases. PR-3, -4, -8, and -11 have been classified as endochitinases. The PR-5 family belongs to the thaumatin-like proteins. PR-6 contains proteinase inhibitors implicated in defense against insects and other herbivores, micro-organisms, and nematodes (Ryan, 1990). PR-7 has so far been characterized only in tomato, where it is a major PR protein and acts as an endoproteinase (Goldman and Goldman, 1998). The PR-9 family consists of peroxidases, which are likely to function in strengthening plant cell walls by catalyzing lignin deposition in reaction to microbial attack. The
PR-10 family is structurally related to ribonucleases (Moiseyev et al., 1997).

The pathogen-induced plant defensins (PR-12), thionins (PR-13), and lipid transfer proteins (LPTs) (PR-14), are families of peptides with antimicrobial activity that have been recently identified in radish (PR-12), Arabidopsis (PR-13) and barley (PR-14).

Not all families of PR proteins have been identified in those plant species examined. In tobacco, for example, PR-7, -10, -12, -13 and -14 are found, suggesting that plant species differ in the type of PR proteins present or, at least expressed upon infection (Van Loon, 1997; Van Loon and Van Strien, 1999).

Since some of the tobacco PR proteins have been identified as chitinases (Legrand et al., 1987) and β-1,3-glucanases (Kauffmann et al., 1987) with potential antifungal activity, it has been suggested that the collective set of PR proteins may be effective in inhibiting pathogen growth, multiplication and/or spread (Stintzi et al., 1993).

### 2.6.5.2 β-1,3-Glucanase

β-1,3-glucanases form part of a general defense system in a number of plant species (for review see Hahn et al. 1989). Many β-1,3-glucanases (EC 3.2.1.39) have been purified and characterized. Like most of the PR proteins, they usually are monomeres with a molecular mass in the 25-35 kDa range (Stintzi et al., 1993). Most of these enzymes are endoglucanases, producing oligomers of chain lengths 2-6 glucose units from the classical substrate laminarin, an almost unbranched β-1,3-glucan (Stintzi et al., 1993).

In healthy plants high concentrations of β-1,3-glucanases accumulate in the roots and lower leaves (Felix and Meins, 1986). These enzymes
are also present in specialized tissues, e.g. the leaf epidermis (Keefe et al., 1990) and stylar tissue (Lotan et al., 1989). Combinations of auxin and cytokinin regulate these enzymes and their mRNAs in cultured tobacco cells (Mohnen et al., 1985; Felix and Meins, 1986). According to Kauffman et al. (1987) and Kombrink et al. (1988) $\beta$-1,3-glucanase became more active when responding to pathogen interaction or hormonal treatments in higher plants.

2.6.5.3 Chitinase

Similar to the $\beta$-1,3-glucanases several chitinases (EC 3.2.1.14) have been purified and characterized. They usually are monomers of 25-35 kD molecular mass. Most plant chitinases characterised so far are endochitinases, producing chito-oligosaccharides of 2 to 6 N-acetylglucosamine units. Many purified plant endochitinases also possess some lysozyme activity (Stintzi et al., 1993).

Chitin is a $\beta$-1,4-linked polymer of N-acetylglucosamine and a structural component for a wide range of organisms, including fungi, crustacean and nematode eggs, and insects (Gooday, 1990; Flach et al., 1992; Cohen, 1993). According to Roberts (1992), chitin forms a complex with various other substances, like polysaccharides and proteins.

Micro organisms such as soil bacteria (Ordentlich et al., 1988) and various fungi (Lorito et al., 1993) also secrete chitinases. Increased chitinase activity in response to fungal, bacterial and viral infections have been reported for various monocotyledonous and dicotyledonous plants (for review see Punja and Zhang, 1993).

There are differences in specific and antifungal activity of the various chitinases from a given plant species (Huynh et al., 1992; Sela-Buurlage et al., 1993). For example, acidic (class II or class III)
chitinase taken from tobacco or chickpea displayed less antifungal activity when tested in vitro than basic chitinase (Sela-Buurlage et al., 1993).

2.6.5.4 The effect of chitinase and $\beta$-1,3-glucanase on plant pathogens

The current interest in $\beta$-1,3-glucanases and chitinases stems from the hypothesis that these hydrolases play an important role in plant defense. Since many fungi contain chitin and $\beta$-glucan as a major structural component of their cell walls (Wessels and Sietsma, 1981), it has been suggested that $\beta$-1,3-glucanases and chitinases may defend the plants against infection by pathogenic and potentially pathogenic fungi (Abeles et al., 1971; Pegg, 1977; Boller, 1985; Bowles, 1990).

This suggestion has been supported by in vitro experiments in which erosion of fungal cell walls caused by $\beta$-1,3-glucanases and chitinases leads to lysis of the hyphal tip, especially when these two enzymes act in combination (Mauch et al., 1988; Sela-Buurlage et al., 1993; Ji and Kuc, 1996). Furthermore, it has been shown that the released glucan and N-acetylglucosamine fragments may act as elicitors of host defense responses (Keen and Yoshikawa, 1983; Kurosaki et al., 1988; Yoshikawa et al., 1990).

Studies on the subcellular localization of chitinases and $\beta$-1,3-glucanases in planta provide additional information about the role of these hydrolytic enzymes during the active defense. A considerable amount of chitinase and $\beta$-1,3-glucanase has been found in the intercellular space of stem and leaf rust infected wheat leaves (Sock et al., 1990; Hu and Rijkenberg, 1998) and Cladosporium fulvum infected tomato leaves (Wubben et al., 1992).
Recently, substantial evidence has been obtained that support the antifungal role of chitinases and β-1,3-glucanases \textit{in vivo}. Constitutive expression of chitinases and β-1,3-glucanases enhance the resistance of tomato to \textit{Alternaria solani} (Lawrence \textit{et al.}, 1996). Transgenic plants constitutively expressing chitinases and β-1,3-glucanases, show significantly increased protection against fungal pathogens (Zhu \textit{et al.}, 1994). The hybrid \textit{Nicotiana glutinosa} X \textit{N. debneyi}, which constitutively expresses high levels of chitinases, β-1,3-glucanases, peroxidases, and polyphenoloxidase, shows increased resistance against viral, bacterial and fungal infections compared with either parental species (Ahl and Gianinazzi, 1982).

### 2.6.5.5 Peroxidase

Peroxidases (EC 1.11.1.7) are proteins that can change the properties of the extracellular matrix. These enzymes have been studied extensively in higher plants, and their activity and isoenzyme pattern can be correlated with a number of growth, developmental, and defense processes (Bowles, 1990).

Peroxidases have been implicated as a contributing factor in resistance against both pathogens and insects (Hammerschmidt \textit{et al.}, 1982; Edreva \textit{et al.}, 1989; Stout \textit{et al.}, 1990; Molinari, 1991; Van der Westhuizen \textit{et al.}, 1998). Increased peroxidase activity has also been reported after infection with different fungi, including \textit{P. recondita} (Johnson and Cunningham, 1972) and \textit{P. graminis} (Moerschbacher \textit{et al.}, 1966; 1988). According to the latest classification of PR proteins proposed by Van Loon and Van Strien (1999), the peroxidases belong to the PR-9 family of PR proteins, which are involved in strengthening plant cell walls. Stress stimuli of different sources, including wounding (Lagrimini and Rothstein, 1987), light (Casal \textit{et al.}, 1994), low CO\textsubscript{2} concentration (Takeda \textit{et al.}, 1993), application of ethylene (Indemarsson, 1995), sodium chloride (Mittal and Dubey, 1991),
abscisic acid (Chaloupkova and Smart, 1994), and indole acetic acid (Birecka and Miller, 1974) have been reported to induce peroxidase activity.

Peroxidases are involved in four main events that occur in the extracellular matrix, namely oxidative burst, lignin synthesis, suberin synthesis, and the construction of intermolecular linkages (Bowles, 1990).

2.7 DISEASE CONTROL
Wheat rust can be controlled by several methods, none of which is completely satisfactory on its own. The earliest attempts to control wheat rust involved religious practices (Roelfs et al., 1992). These ceremonies existed as early as 1000 B.C., continued into the first century A.D., and apparently varied between geographic areas. In the early 1600s, Worldridge (Roelfs, 1985) recommended pulling a rope over the grain to reduce dew deposition on plants. This practice continued until the 1900s in some areas, while in France, according to Roelfs (in Knott, 1989), the laws required barberry eradication in 1660. Today, rust is primarily controlled genetically or by the use of chemicals, and to a lesser extent by cultural methods (Knott, 1989).

2.7.1 Breeding for resistance
2.7.1.1 General approach
Breeding for disease resistance is an effective way of protecting crops from damage due to biotic factors. Wheat rust fungi, like most organisms, are prone to mutation and recombination which lead to new pathogen races that can overcome host plant resistance. Scientists are continually searching for new resistance genes in order to increase the genetic diversity of wheat (McIntosh et al., 1995).
Regardless of the individual viewpoints on the development and management of disease resistance, the first requirement for the breeder is a source of resistance, or the means of developing a level of resistance that results in less disease and/or a reduction in crop losses. Following the identification of a resistance source, genetic studies are undertaken to determine the components of resistance and their phenotypic and genetic characteristics. These studies are usually independent of resistance breeding because the choice of susceptible parents is based on different criteria. Following these studies, decisions can be taken on the advisability of, and the optimal strategy for, commercial exploitation.

Genetic studies of resistance sources usually proceed in several stages, often beginning with the identification of resistance among the materials comprising a field rust nursery. The next step is to determine if the resistance is effective at the seedling stage and whether it is effective against a wide array of pathotypes. From the pedigree and multi-pathotype seedling tests it may be possible to postulate the presence of known genes in candidate lines and to decide whether a genetic study is worthwhile.

Genes for rust resistance are named after the first letters of the common name for the disease. Numbers are used to specify specific genes and lower case letters designate alleles (Knott, 1989). To date, several different stem rust (Sr), stripe rust (Yr), and leaf rust (Lr) resistance genes have been characterized (McIntosh et al., 1995).

According to Gerechter-Amitai and Stubbs (1970) the Yr15 gene on which this study is based, was transferred from T. turgidum var. dicoccoides (wild emmer) accession G25. Gerechter-Amitai et al. (1989) noted that Yr15 is present in other collections of wild emmer.
McIntosh et al. (1995) also noted that resistance was transferred from G25 to cultivated tetraploid and hexaploid wheats.

Wheat lines with Yr15 are resistant to most isolates of *P. striiformis* f. sp. *tritici* but virulence has been reported from Afghanistan and other locations (McIntosh et al., 1995). According to the latter authors, Yr15 is not currently in use in commercial cultivars, although the gene has been used in wheat breeding. McIntosh et al. (1996) showed that Yr15 is located in chromosome 1BS and that it is linked (34 cM) to Yr10. Despite the highly resistant response mediated by Yr15, McIntosh et al. (1996) were of the opinion that its resistance will not be durable. They suggested that the gene be combined in backgrounds with adult-plant resistance, but recognised that confirmation of the latter may be difficult in the presence of the immune response of Yr15.

Although considerable advances have been made in conventional breeding for rust resistance, the availability of molecular techniques provides the breeder with additional selection tools.

### 2.7.1.2 Plant breeding strategies and molecular markers

The aim of plant breeding programmes is the improvement of crop plants by combining desirable characters present in different breeding lines. This is achieved by crossing potential donor lines possessing the desirable trait, with recipient plants that already possess other important traits. This process is rather time-consuming and involves repeated backcrossing or selfing and selection for the desired traits. Resistance genes are often lost in segregating populations, due to insufficient selection techniques (Kelly, 1995). The main objective of a selection programme for any monogenic plant trait is to increase the proportion of individuals that are homozygous for a particular favourable allele (Michelmore, 1995). In the case of a dominant allele, progeny testing has to be performed after direct selection of the allele.
in order to distinguish homozygous and heterozygous genotypes. Conversely, after direct selection for a recessive allele, the particular locus is immediately fixed in the homozygous recessive state. Molecular markers can be used to accelerate the generation of homozygosity by allowing plant breeders to distinguish between homozygous and heterozygous genotypes (Kelly, 1995).

The efficiency of a breeding programme lies in the methods available to detect desirable traits (Winter and Kahl, 1995). Adequate trait detection is not always available to plant breeders. Complex plant traits encoded by multiple genes are difficult to select for, due to the phenotypic absence of markers. Furthermore, selection for multiple genes is more difficult than single genes as a result of additive effects. The selection of recessive genes is also difficult to achieve using classical methods (Winter and Kahl, 1995). In contrast to human and forensic genetic applications, which require high accuracy for a number of established assays, plant breeding programmes require a molecular diagnostic assay that is relatively inexpensive and can be performed on many individual plants with an accuracy of 95% (Rafalski and Tingey, 1993).

Marker-assisted selection (MAS) (Melchinger, 1990) can be used to increase the efficiency of selection in plant breeding. However, before using molecular markers in a breeding programme, it is important to consider the characteristics of the genetic marker system and the structure of the breeding population being tested (Kelly, 1995).

The efficiency of MAS is dramatically influenced by the linkage distance between the marker and the gene of interest. This distance can vary substantially in different genetic backgrounds. For example, the RAPD OPA14,1000 marker for Up-2 Andean rust resistance in
common bean could only be used for detection of the gene in the Middle American gene pool (Haley et al., 1994).

In crossing a wild-type cultivar with a commercial line whole chromosome fragments are translocated, rather than just specific genes. This process is known as linkage drag (Stam and Zeven, 1981; Zeven et al., 1983). The observation of the tight linkage of inter-gene pool or wide crosses has led to the analysis that linkage drag plays a more important role than where intra-gene pool crosses are concerned. Linkage drag results in an over-estimation of linkage distances in gene tagging and map construction (Paterson et al., 1990). This results in markers which are ineffective in inter-species crosses. The most useful markers are, therefore, those that are closely linked to the gene of interest across a broad range of genetic backgrounds (Kelly, 1995).

Using a wide range of molecular techniques to tag genes, it is now possible to accelerate the transfer and introgression of novel genes from related wild species. Polygenic characters which are difficult to analyse using traditional methods, can also be tagged. Furthermore, these techniques are also useful to determine genetic relationships between sexually incompatible crop plants.

Techniques which are used to identify genetic markers for desirable characters include Random Amplified Polymorphic DNA Markers (RAPDs) (Williams et al., 1990), Restriction Fragment Length Polymorphisms (RFLPs) (Staub et al., 1996), Sequence Characterised Amplified Regions (SCARs) (Paran and Michelmore, 1993), Sequence Tagged Sites (STGs) (Olson et al., 1989), Simple Sequence Repeats (SSRs) (Zietkiewicz et al., 1994), microsatellites (Staub et al., 1996) and Amplified Fragment Length Polymorphic DNA (AFLPs) (Mohan et al., 1997).
RFLPs are detected by the use of restriction enzymes that cut genomic DNA at specific nucleotide sequences (restriction sites), thereby yielding variable-size DNA fragments. Visualisation of these fragments yield RFLPs, which are codominant markers (Staub et al., 1996). RAPD markers are generated by PCR amplification of random genomic DNA segments with primers of arbitrary sequence (Williams et al., 1990). RAPDs are usually dominant markers with polymorphisms between individuals defined as the presence or absence of a particular RAPD band. A RAPD marker linked to a particular gene can be developed into a PCR-based assay called a SCAR by sequencing the ends of the amplified fragment (Paran and Michelmore, 1993). Such SCARs are similar to sequence tagged-sites (STS) in construction and application (Olson et al., 1989). Sequence tagged-sites are a similar further development of an RFLP fragment. Microsatellites are comprised of tandem arrays of 2-5 base pair monomeric repeat units. Polymorphisms are detected as a result of variation in the number of tandem repeats in a given repeat motif. Most SSRs are dinucleotide repeat-based microsatellites. Microsatellite DNA sequences are excellent sources of polymorphisms in eukaryotic genomes, and are well suited for genotyping and map construction (Staub et al., 1996).

Several molecular markers have been reported for rust resistance genes (Schachermayr et al., 1994; Sun et al., 1997; Gold et al., 1999; Seyfarth et al., 1999). Schachermayr et al. (1994) and Seyfarth et al. (1999) used RFLPs to detect a polymorphic marker for Lr9 and Lr35, respectively. The detected markers were further developed into STS markers. Sun et al. (1997) described two molecular markers, OPB13 and Nor1, flanking Yr15 by 27.1 cM and 11.0 cM, respectively. These markers were produced using RAPD and RFLP methodology. Gold et al. (1999) made use of SCARs to develop a molecular marker
for rust resistance genes \textit{Sr39} and \textit{Lr35}. This marker was present in six wheat lines carrying the \textit{Sr39} and \textit{Lr35} genes on the translocated chromosome segment.

Recently the AFLP fingerprinting technique was developed by Keygene, Inc. This technique selectively amplifies DNA fragments after DNA digestion and ligation of adapters (Zabeau and Vos, 1993) (European Patent Application No. 0534858 A1). Genomic DNA is digested with two endonucleases and site specific adapters are then ligated to the DNA fragments. Primers (complementary to the adapters and the restriction sites) with selective nucleotides added to the 3’ ends of the primers are used to amplify selective fragments. Only DNA fragments which complementary match the selective nucleotides of the primer are amplified during PCR. Resulting fragments are resolved on standard sequencing gels or using automated sequencing.

The advantage of AFLPs is that the assay requires no prior sequence knowledge, and it detects a 10-fold greater number of loci (20-100) than RAPDs (Maughan \textit{et al.}, 1996). The capacity to screen large numbers of loci lends AFLPs to linkage mapping on near-isogenic lines (Muehlbauer \textit{et al.}, 1988; Young \textit{et al.}, 1988) and bulk segregant analysis (Michelmore \textit{et al.}, 1991).

In conclusion, by using AFLP analysis it is possible to detect large numbers of genetic loci in a single reaction. Although more costly than RAPDs, AFLP analysis is a quick, robust technique which, unlike RFLPs, requires minimal preliminary work. Furthermore, AFLPs detect dominant and co-dominant markers. Undoubtedly, these characteristics make the AFLP technique an excellent method for the detection of genetic markers in a wide variety of plant species (Maughan \textit{et al.}, 1996).
2.7.2 Cultural methods

Cultural methods of rust control are aimed at disrupting the life cycle of the fungus at a critical stage (Roelfs, 1985). In the U.S.A. cultural methods depend largely on the use of early maturing cultivars and the planting of spring wheats. Furthermore, early planting may increase the chance for infection during autumn and subsequent overwintering of the rust in milder climates of the U.S.A. (Roelfs, 1985). In Australia, Farrer developed early-maturing wheat cultivars to escape the damage caused by rust (McIntosh, 1976).

Most of the environmental conditions that favour wheat also favour rust development (Roelfs, 1985). Zadoks and Bouwman (1985) highlighted the importance of the green bridge in carrying the disease from one crop to the next. When some farmers plant early and others late, the green bridge is extended (Roelfs et al., 1992). Any successful efforts in removing the green bridge, either with tillage or herbicides, may help in preventing damaging epidemics (Roelfs et al., 1992). In areas where rust oversummers, the destruction of volunteer wheats and susceptible grasses several weeks before planting reduces the inoculum density, and therefore delays the initial infection (Roelfs, 1985). When both spring and winter wheat are grown in the same area, the separation of these crops by space or another non-susceptible crop can delay disease spread between fields (Roelfs, 1985).

2.7.3 Chemical control

Studies on chemical control of cereal rusts began in the last century, but Dickson (Samborski, 1985) concluded it was not economically viable. Chemicals are expensive, an expenditure compounded by the added cost of application. According to Knott (1989) fungicide application is only economical where intensive cereal management is
practised and where a complex of diseases can be controlled. Chemicals have been successfully used in Europe where high yields (6 to 7 t/ha) are regularly achieved (Buchenauer, 1982). Chemical control is especially valuable when new rust races develop and resistant cultivars are not available (Samborski, 1985).

Fungicides are usually important in the less developed countries, but may not be readily available. Farmers cannot afford to buy chemicals or the equipment to apply them, and therefore it is not a viable method for rust control in these countries (Knott, 1989).

It has been estimated that in 1996 alone R30 million was spent on chemicals to control stripe rust in the Western Cape. Likewise, an estimated R18 million was spent on stripe rust control in the Eastern Free State in 1997. Although acceptable control was achieved in many areas, several questions arose pertaining to the efficacy of some of the compounds, the timing of applications, susceptibility of cultivars and the economic benefits of using fungicides (Lochner, 1998). Several fungicides have been registered for stripe rust control in South Africa (Nel et al., 1999).

2.7.4 Conclusion
From the literature it is clear that breeding for resistance is the most economical and environment-friendly measure to control rust diseases of cereals. By characterizing resistance conferred by Yr15, it is hoped that this information may assist wheat breeders to achieve genetic control of stripe rust.
Chapter 3

HISTOPATHOLOGY OF Yr15 RESISTANCE IN WHEAT TO STRIPE RUST

3.1 INTRODUCTION

The development of specialised fungal infection structures, e.g. the germ tube, appressorium, infection peg, substomatal vesicle and infection hyphae, is characteristic of a rust species and important for successful entry and colonisation of a host plant (Littlefield and Heath, 1979). This has led to the description of several mechanisms of resistance in plants to rust pathogens. Following infection by a pathogen, most higher plants employ the hypersensitive response, characterised by rapid cell death at the infection site, as an effective defense system (Keen, 1990). Prehaustorial and posthaustorial resistance have also been identified as broad categories indicating the time of resistance onset (Heath, 1981; Niks and Dekens, 1991).

Histological studies for several plant pathogens have been reported (Napia-Cedo and Exconde, 1965; Leath and Rowell, 1966; Miller et al., 1966; Kraft et al., 1967; Spencer and Cooper, 1967; Heath, 1971; Nemec, 1972; Dow and Lumsden, 1975; McKeen, 1977; Strömberg and Brishammar, 1992; Kroes et al., 1998; Metcalf and Wilson, 1999). More specifically, germination and histopathological aspects of stripe rust infection have been studied by Marryat (according to Mares and Cousen, 1977), Sharp (1965), Mares and Cousen (1977), Mares (1979), Rubiales and Niks (1992), De Vallavieille-Pope et al. (1995) and Broers and López-Atilano (1996).

Using Yr15 as a resistance source, the mode of penetration on wheat seedlings by P. striiformis f. sp. tritici, as well as the development of the fungus in its host, formed the basis of this investigation. Specific objectives
were to investigate the feasibility of microscopy techniques used in other cereal rust pathosystems, as well as to contrast the infection process between wheat lines resistant and susceptible to stripe rust.

3.2 MATERIALS AND METHODS

3.2.1 Host genotypes
The expression of stripe rust resistance was studied in the bread wheat line Yr15/6*AvS. The wheat cultivar Avocet'S' (AvS) was included as the stripe rust-susceptible control. Seeds of both lines were obtained from the Department of Plant Pathology, University of the Orange Free State, Bloemfontein. Two replicate pots per genotype were sown. Plants were grown in a stripe rust-free air-conditioned glasshouse cubicle where a day/night temperature cycle of 22 to 25°C/16 to 18°C was maintained. Natural daylight was supplemented with 120 μmolm⁻²s⁻¹ photosynthetically active radiation emitted by cool-white fluorescent tubes, arranged directly above plants, for 14 h each day.

3.2.2 Inoculation and incubation
Prior to inoculation, urediniospores of the stripe rust pathotype 6E16 was multiplied in isolation on seedlings of Morocco wheat. One day before inoculation the seedlings were acclimatised at 16°C. Seven-day-old seedlings were spray-inoculated (approximately 2 mg spores/ml oil) with freshly collected spores suspended in light mineral oil and kept in darkness in a dew chamber at 7 to 9°C for 30 h. Seedlings were then placed at 18°C in a growth chamber where 14 h of 200 μmolm⁻²s⁻¹ photosynthetically active radiation, emitted by cool-white fluorescent tubes and arranged 30 cm above plants, was provided.

3.2.3 Fluorescence microscopy
Three primary leaves of each genotype were randomly sampled from each of the two replicates 30 h post-inoculation (30 hpi), six days post-inoculation (6
dpi) and again 13 days post-inoculation (13 dpi). Two-centimetre primary leaf sections were prepared for fluorescence microscopy (Rohringer et al., 1977; Kuck et al., 1981). Uvitex 2B (Novartis, Basel, Switzerland) (Niks and Dekens, 1991) was used as the fluorescent stain. Observations were carried out at X100 or X400 with a Nikon Labophot epifluorescence microscope, using the filter combination UV-1A (excitation filter 330-380 nm and barrier filter 420 nm) for fungal structures and B-2A (excitation filter 450-490 nm and barrier filter 520 nm) for observations of plant cell necrosis.

Fungal structures were classified into the following categories: non-penetrating germ tubes, aborted substomatal vesicles (ASSV), aborted substomatal vesicles with necrosis (ASSVN), early abortions (EA) (six or less haustorium mother cells per infection site), early abortions with necrosis (EAN), and colonies (Niks, 1987). Parlevliet and Kievit (1986) classified abortive penetration (AP) as sporelings that did not develop beyond the substomatal vesicle phase. The number of haustorium mother cells was counted at X100 magnification and confirmed at X400 where necessary.

3.2.4 Phase contrast microscopy
Two leaves of each wheat line were sampled at 30 hpi and 6 dpi and then cut into 2-cm segments. Leaf segments were cleared and fixed in ethanol:dichloromethane (3:1 v/v) + 0.15% trichloroacetic acid for 24 h before they were boiled for 5 min in a 0.03% solution of trypan blue in lactophenol:ethanol (1:2 v/v). Specimens were then cleared by immersing them for 24 h in a saturated solution of chloral hydrate (5:2 m/v) and stored in 50% glycerol with a trace of lactophenol.

To determine whether cell wall appositions are formed as part of the Yr15 defense response, specimens were transferred through a series of 80% (30 min), 90% (30 min) and 100% (2 X 30 min) ethanol for dehydration. Thereafter they were stained with a saturated solution of picric acid in methyl salicylate for 5 min (Niks, 1986). Stained segments were mounted with
adaxial sides upwards in methyl salicylate under coverslips sealed with nail varnish.

Leaf segments were used as whole mounts for phase contrast microscopy. Screening of leaf segments for detection of infection sites was conducted at X100. Detailed observation of haustoria and cell wall appositions were conducted at X1000 (oil immersion).

### 3.2.5 Scanning electron microscopy

Three primary leaves of each genotype were randomly sampled from each replicate at 72 hpi and 7 dpi. Leaves were cut into 5 mm sections and fixed in 3% gluteraldehyde. Leaf pieces were washed twice in 0.05 M phosphate buffer and post-fixed in 2% osmium tetroxide. The phosphate buffer consisted of 0.2M Na$_2$HPO$_4$ (30.5 ml) and 0.2M NaH$_2$PO$_4$ (19.5 ml), adjusted to 100 ml with distilled water. Both fixatives were dissolved in 0.05% phosphate buffer (pH = 6.8 to 7.2). The material was then dehydrated through an acetone series and critical-point dried in a Polaron critical point dryer. All specimens were gold/palladium coated in a Bio-Rad SEM coating system and viewed with a JEOL WINSEM JSM-6400 scanning microscope operating at 5kV.

Fungal behaviour on the leaf surface was classified into uninfected stomata and stomata which appeared to be successfully penetrated by a germ tube or network of germ tubes. To investigate fungal structures inside the leaf, the epidermis was stripped using the technique described by Hughes and Rijkenberg (1985). Stomata were classified as either penetrated or uninfected. Observations of aborted substomatal vesicle initials, substomatal vesicles, and primary infection hyphae were also made.

### 3.2.6 Data analysis
The statistical program NCSS2000 (Statistical Solutions, Cork, Ireland) was used for analysis of variance and for calculating standard deviations of treatment means.

3.3 RESULTS

3.3.1 Fluorescence microscopy

Histological data indicating the relative proportions of fungal structures, their abortion and association with host cell necrosis, in resistant (Yr15) and susceptible (AvS) wheat seedlings, are summarised in Fig. 3.1 and Table 3.1. According to the statistical analyses no significant differences were observed between the resistant and susceptible lines for AP and EA (Fig. 3.1). Significantly (P<0.05) more necrosis was associated with ASSV and EA in the Yr15 line than in AvS (Table 3.1).

Non-penetrating germ tubes
At 30 hpi and 6 dpi more than 90% of germinated spores did not grow over or penetrate stomata on both lines (Fig. 3.1). At 13 dpi a similar proportion of non-penetrating germ tubes was observed for Yr15. At this sampling time, however, only 33.9% of germ tubes did not penetrate stomata on AvS.

Aborted substomatal vesicles
At 30 hpi 6.5% and 4.3% of the infection sites studied in Yr15 and AvS were classified as AP, respectively (Fig. 3.1). Of these, none was associated with host cell necrosis (Table 3.1). At 6 and 13 dpi the proportion of sites with AP on Yr15 was 2.8% and 3.4%, respectively. The frequency of AP on the susceptible line was statistically similar to that of Yr15 at 6 dpi, whereas at 13 dpi the extensive mycelial growth in AvS did not allow differentiation of ASSV. Approximately 40% of sites with ASSV on Yr15 showed host cell necrosis at 6 and 13 dpi (Table 3.1). No necrotic cells were observed for AvS at 6 and 13 dpi.
Fig. 3.1. Histological data indicating relative proportions of fungal structure development in resistant (Yr15) and susceptible (AvS) wheat seedlings sampled at different hours (h) and days (d) post-inoculation (pi). Data represent the relative proportion of infection sites displaying the above stages of fungal development.
Table 3.1. The association of host cell necrosis with infection sites displaying either aborted substomatal vesicles or early abortion of *Puccinia striiformis* f. sp. *tritici* in resistant (Yr15) and susceptible (AvS) wheat lines, using fluorescence microscopy

<table>
<thead>
<tr>
<th></th>
<th>Percentage sites with necrosis</th>
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<tbody>
<tr>
<td></td>
<td>30hpi</td>
</tr>
<tr>
<td></td>
<td>AvS</td>
</tr>
<tr>
<td>Abortive penetration</td>
<td>0</td>
</tr>
<tr>
<td>Early abortion</td>
<td>0</td>
</tr>
</tbody>
</table>
Early abortion
EA could not be detected at 30 hpi. In the resistant line only a small percentage of sites showed EA (2.5% at 6 dpi and 2.4% at 13 dpi) (Fig. 3.1), with 50% of these sites associated with necrosis (Table 3.1) (Fig. 3.2). A small percentage of sites (3.7%) was misclassified as early abortions in AvS at 6 dpi, as the 13 dpi sampling showed that all sites eventually developed into colonies. No necrosis was associated with AvS at 6 and 13 dpi.

Colonies
A high proportion (66.1%) of colonies (sites with more than six haustorium mother cells), all without any host cell necrosis, occurred in AvS at 13 dpi (Fig. 3.1). According to this criterion no colonies were observed in the resistant line.

3.3.2 Phase contrast microscopy
Observations of haustoria and possible cell wall appositions were made at 6 dpi only. The 30 hpi sampling was considered too early to be representative of eventual fungal development and at 13 dpi mycelial colonisation prevented haustorium counts in AvS. Three hundred and six infection sites were studied in the resistant line and 123 in AvS.

Using the trypan blue staining method, substomatal vesicles, infection hyphae, haustorium mother cells, and haustoriums could be observed (Fig. 3.3A).

Number of haustoria per colony
The susceptible line showed a significant (P<0.05), ten fold increase in the mean number of haustoria (Fig. 3.3B) per colony when compared to Yr15 (Fig. 3.4). In the resistant line haustoria were observed in about 35% of the infection sites studied, and in most cases only one haustorium was visible (Fig. 3.4). No cell wall appositions were detected.
Fig. 3.2. An infection site of pathotype 6E16 of *Puccinia striiformis* f. sp. *tritici* on Avocet'S' 6 days post-inoculation, showing (A) a substomatal vesicle (SSV) and haustorium mother cells (HMC) as viewed with fluorescence microscopy filter combination UV-1A, and (B) associated host cell necrosis at the same site, using the B-2A filter.
Fig. 3.3. Hyphal growth and haustorium mother cell formation in primary Avocet'S' leaves inoculated with pathotype 6E16 of *Puccinia striiformis* f. sp. *tritici* (A), stained with trypan blue 6 days post-inoculation, and (B) haustoria of pathotype 6E16 in host cells of Avocet'S’ stained with picric acid and methyl salicylate. Abbreviations used: SSV = substomatal vesicle; IH = infection hypha; H = haustorium; HN = haustorium neck.
Fig. 3.4. Number of haustoria per colony of *Puccinia striiformis* f. sp. *tritici* for resistant (*Yr15*) and susceptible (*AvS*) wheat lines 6 days post-infection. Error bars indicate the standard deviation.
3.3.3 Scanning electron microscopy
In general the percentage infected stomata, viewed from the upper leaf surface, was similar for both the resistant and susceptible lines at both sampling stages (Table 3.2). In this investigation only stomata where germ tubes appeared to have successfully entered, were considered infected. Numerous stomata were overgrown by germ tubes or a network of tubes (Fig. 3.5A). In many cases germ tubes grew transversally over the leaf, turning parallel to the opening when a stoma is reached (Figs. 3.5B and C), suggesting some form of recognition. However, the collapsed state of most germ tubes did not provide a clear indication of stomatal penetration.

Based on the frequency of substomatal vesicles or their initials (Fig. 3.6), it appeared that very few stomata (1.8 to 3.2%) were penetrated (Table 3.2). Similar to the observations on the leaf surface, no clear differences between Yr15/AvS or AvS were detected.

3.3.4 Infection types
The infection types of Yr15/AvS and AvS to pathotype 6E16 at full symptom development are presented in Fig. 3.7.

3.4 DISCUSSION
In a favourable environment the urediniospore of the stripe rust fungus hydrates, swells, and germinates through a germ pore to form a germ tube. When colonising a living wheat plant, the germ tube penetrates its host via a stoma, and invades the leaf tissue by developing a set of specialised infection structures (Rohringer and Heitefuss, 1984). Mares and Cousen (1977) described this process and showed diagrammatically how a poorly defined appressorium forms over a stoma to produce the substomatal vesicle and infection hyphae. When these primary hyphae reach a mesophyll cell, a septum forms, delimiting the haustorium mother cell which then penetrates the host cell to form an intracellular haustorium. This is often accompanied
Table 3.2. Frequency of stomatal penetration in wheat lines infected with pathotype 6E16 of *Puccinia striiformis* f. sp. *tritici*, using scanning electron microscopy

<table>
<thead>
<tr>
<th>Wheat line/ sampling time</th>
<th>Upper surface(^a) (%)</th>
<th>Inner surface(^b) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>72hpi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yr15/6*AvS</td>
<td>3.92 ± 1.85</td>
<td>2.17 ± 1.32</td>
</tr>
<tr>
<td>Avocet'S'</td>
<td>4.15 ± 1.09</td>
<td>3.23 ± 0.38</td>
</tr>
<tr>
<td>7dpi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yr15/6*AvS</td>
<td>1.09 ± 0.84</td>
<td>1.81 ± 0.10</td>
</tr>
<tr>
<td>Avocet'S'</td>
<td>1.79 ± 0.75</td>
<td>2.23 ± 0.70</td>
</tr>
</tbody>
</table>

\(^a\) A minimum of 893 stomata were examined on the leaf surface.

\(^b\) A minimum of 305 stomata were viewed from the inner epidermal surface.
Fig. 3.5. Scanning electron micrographs of (A) a network of germ tubes of pathotype 6E16 of *Puccinia striiformis* f. sp. *tritici* on the leaf surface of Avocet 'S', (B) germ tube orientation upon contact with stomatal opening, and (C) continued and redirected growth of germ tube after stomatal contact. Abbreviations used: G = germ tube; S = stoma, U = urediniospore. Scale bar represents $10\mu m$. 
Fig. 3.6. Scanning electron micrographs of (A) substomatal vesicle initial, (B) substomatal vesicle and (C) vesicle with primary infection hyphae and haustorium mother cell of pathotype 6E16 of *Puccinia striiformis* f. sp. *tritici*. Structures were viewed from the inner epidermal surface of Avocet’S’ sampled 30 h post-inoculation. Abbreviations used: SSVI = substomatal vesicle initial; SSV = substomatal vesicle; PIH = primary infection hypha; HMC = haustorium mother cell. Scale bar represents 10μm.
Fig. 3.7. Infection types produced by pathotype 6E16 of *Puccinia striiformis* f. sp. *tritici* on primary leaves of (A) Yr15/6*AvS* and (B) Avocet'S'.

by necrosis or death of invaded and neighbouring cells, resulting in macroscopic lesions at the infection sites.

Using fluorescence microscopy, Broers and López-Atilano (1996) reported the formation of appressoria of *P. striiformis* f. sp. *tritici* on wheat. They did not describe the actual morphology of the appressoria, but found that the occurrence thereof was infrequent (<26% of stomata covered). Evidence was however presented that appressoria occurred more frequently on the susceptible control Morocco, than on the resistant cultivars. Earlier, Sharp (1965) also considered a swelling at the end of the germtube as an appressorium. Mares and Cousen (1977) noted that germ tubes of the stripe rust fungus penetrated stomata after forming a “weak appressorium”. They found that 25 to 30% of germ tubes actually penetrated stomata. In the present study no evidence of appressoria, irrespective of the microscopy technique used, was obtained. Similarly, Rubiales and Niks (1992) and De Vallaieille-Pope *et al.* (1995) stated that *P. striiformis* penetrates its hosts without forming an appressorium. Germ tubes often appeared collapsed and those sections that touched or overgrew stomata did not appear to be differentiated into a distinct appressorium. An explanation could be that leaves were sampled after appressoria had already collapsed. The study of Broers and López-Atilano (1996) showed, however, that more appressoria were encountered at 96 and 144 hpi than at 48 hpi.

Scanning electron microscopy of the leaf surface often showed a network of germ tubes. Although this created the impression of anastomosed germ tubes, closer examination did not confirm this. These networks were not visible using fluorescence or phase contrast microscopy.

Similar to previous work (Mares and Cousen, 1977; Broers and López-Atilano, 1996) the frequency of stomatal penetration was extremely low in the present study. In the *P. recondita* f. sp. *tritici* - wheat system, prestomatal exclusion of approximately 60% (Bender, 1995), 35% (Jacobs, 1996) and 11
to 50% (Barnard, 1999) has been reported. According to Rubiales and Niks (1992) less than 60% of *P. striiformis* spores germinated and, of these, more than 70% of the germ tubes did not end on a stoma. De Vallavieille-Pope *et al.* (1995) also reported that the infection efficiency of *P. recondita* f. sp. *tritici* was 12 times greater than for *P. striiformis* f. sp. *tritici*. No meaningful differences between the resistant *Yr15* and susceptible AvS lines were encountered for infection frequency at the first two sampling times. An obvious explanation for the significantly reduced number of non-penetrants on AvS sampled 13 dpi is that these germ tubes had been dessicated on the leaf surface and were destroyed during the staining procedure. Mares (1979) similarly did not detect differences between susceptible and resistant wheats prior to haustorium formation.

Using scanning electron microscopy, data obtained for infection frequencies on the upper leaf surface also corresponded with that for the inner surface. Since many germ tubes were seen to overgrow stomata, the question was raised whether these could actually penetrate and continue its development on the leaf surface. However, enumeration of substomatal initials and vesicles did not show a higher frequency of infection than that observed from the upper leaf surface. Rubiales and Niks (1992) suggested that *P. striiformis* differs from other cereal rusts in recognizing stomata, but that their data were inconclusive in providing an explanation.

The fluorescence microscope was useful in observing pathogen growth on the leaf surface. The blue-coloured spores and germ tubes were easy to detect. The hypersensitive reaction of *Yr15*, as confirmed by the accompanying death of host cells, was equally visible by switching to the B-2A filter. Vesicles formed in the substomatal cavities were discernible with all microscopy techniques used. However, fluorescence microscopy appeared more suitable to quantify vesicles and subsequent fungal differentiation. Only a few vesicles and hyphae with delimited mother cells were seen using the scanning electron microscope. This could be attributed to the scale of
magnification and the apparent low infection frequency of this particular fungus. It is also possible that the fungus, which grows systemically in the host, was destroyed during epidermal stripping. In contrast, Jacobs (1996) found scanning electron microscopy useful in studying the onset of resistance in wheat to leaf rust.

This study supports the observation of Bender (1995) that young haustoria become more visible in host cells after staining with trypan blue, picric acid and methyl salicylate, rather than with trypan blue only. Interestingly, papillae, usually visible as luminous structures, did not seem to be involved in the highly effective resistance response of Yr15 to pathotype 6E16.

Histological evidence was provided that Yr15 conditions a hypersensitive reaction typical of non-durable resistance. Fluorescence, phase contrast and scanning electron microscopy complemented each other and may be used to quantify various aspects of resistance expression in this pathosystem. However, more work is required to determine the optimum sampling time in relation to fungal differentiation.
Chapter 4

THE ROLE OF DEFENSE-RELATED PROTEINS
IN WHEAT STRIPE RUST RESISTANCE
CONTROLLED BY Yr15

4.1 INTRODUCTION

In recent years increasing evidence has been provided that plant hydrolytic enzymes, e.g. β-1,3-glucanases and chitinases, are involved in the defense strategy against fungal infection (Young and Pegg, 1982; Boller, 1987; Van Loon, 1989; Benhamou et al., 1990; Graham and Graham, 1991; Linthorst, 1991). Since many fungi contain β-1,3-glucans and chitin as structural components in their cell walls (Wessels and Sietsma, 1981; Young and Pegg, 1982), it has been suggested that the enhancement of β-1,3-glucanase and chitinase activity after fungal infection might play an important role in this plant defense response (Young and Pegg, 1982; Mauch et al., 1988; Arlorio et al., 1992). This premise has been confirmed by in vitro experiments in which β-1,3-glucanases, in combination with chitinases, inhibited fungal growth by lysis of the hyphal tips (Mauch et al., 1988; Sela-Buurlage et al., 1993; Ji and Kuc, 1996). Furthermore, oligosaccharide fractions, produced after the breakdown of fungal cell walls, have been shown to act as elicitors of defense responses in plants (Keen and Yoshikawa, 1983; Kurosaki et al., 1988).

Immunolocalization studies confirmed the accumulation of β-1,3-glucanases and chitinases in degenerating materials of fungal origin in the extracellular spaces of host plants during different incompatible plant-pathogen interactions (Sock et al., 1990; Wubben et al., 1992; Hu and Rijkenberg, 1998).
Peroxidases have been studied extensively in higher plants for a number of years, and their activity and isoenzyme patterns have been correlated with growth, developmental, and defense processes (Bowles, 1990). Some peroxidases are also implicated in several defense-related events that occur in the extracellular matrix, including the hypersensitive response (Vera-Estrella et al., 1992), the formation of papillae (Stumm and Gessler, 1986), and the polymerization of lignin from monomeric lignols (Ferrer and Barcelo, 1994). Furthermore, peroxidases have been associated with the cross-linking reactions of cell wall proteins, such as hydroxyproline–rich or glycine-rich glycoproteins (Everdeen et al. 1988). As a result of oxidative cross-linking reactions, cell walls may be strengthened and function as physical barriers against invading pathogens. Moreover, the peroxidases are involved in suberinization, which is a common response to wounding (Kolattukudy and Dean, 1974).

β-1,3-glucanase and chitinase are associated with resistance of wheat to different rust fungi including *P. graminis* (Sock et al., 1990; Münch-Garthoff et al., 1997) and *P. recondita* (Hu and Rijkenberg, 1998; Anguelova et al., 1999, Kemp et al., 1999). Involvement of peroxidases in the resistance of wheat to *P. graminis* (Moerschbacher et al., 1986; 1988) and *P. recondita* (Johnson and Cunningham, 1972) has also been demonstrated. However, as far as could be determined, no experimental studies have shown the involvement of these enzymes in resistance to wheat stripe rust, caused by *Puccinia striiformis* f. sp. *tritici*.

The objective of this study was to ascertain whether intercellular β-1,3-glucanase, chitinase and peroxidase activities are involved in Yr15-resistance of wheat to stripe rust.

### 4.2 MATERIALS AND METHODS

#### 4.2.1 Wheat genotypes and growing conditions
The wheat lines Yr15/6AvS (Yr15) and Avocet’S’ (AvS), respectively resistant and susceptible to stripe rust, were grown in 100-mm diameter pots containing a sterilized soil:peatmoss mixture (1:1 v/v), in a rust-free glasshouse cubicle at 18 to 23°C. A 24 h daylength was provided by cool-white flourescent tubes and incandescent bulbs, emitting 120 μmol m\(^{-2}\) s\(^{-1}\) of photosynthetically active radiation, arranged 30 cm above the plants.

4.2.2 Inoculation
Seedlings were inoculated 10 days after planting by spraying primary leaves with freshly harvested spores of *P. striiformis* f. sp. *tritici* pathotype 6E16, suspended in light mineral oil (2 mg spores/ml oil). Control treatments were sprayed with light mineral oil only. Inoculated and control plants were gradually dried before placement in the dark in a dew chamber at 7°C for 30 h. Plants were then transferred to a growth chamber set at 18°C where light intensity was the same as described above.

4.2.3 Infiltration of leaves and preparation of apoplastic fluid
Infiltration was carried out according to Rohringer *et al.* (1983). Leaves from each treatment were sampled at 0, 24, 48, 96, 168 and 336 hours post inoculation (hpi). Sampled leaves were cut into 80 mm long pieces. The cut ends of the leaves were intensely washed in water to remove intracellular contamination due to mechanical wounding. The leaf pieces were then vacuum-infiltrated for 5 min, using 80 ml of a 50 mM Tris buffer (pH 7.8) containing 0.5 mM PMSF and 5 mM 2-mercaptoethanol. To obtain the apoplastic fluid, leaves were centrifuged at 500 g and 0°C with their tips pointing downwards. The collected apoplastic fluid was frozen at -20°C.

4.2.4 Protein determination
The protein concentration in the extracted apoplastic fluid was determined according to Bradford (1976) using the Bio-Rad protein assay with gamma globulin as standard. The Bio-Rad model 3550 Microplate Reader was used for this purpose.
4.2.5 β-1,3-glucanase assay
A modified method of Fink et al. (1988) was used. A standard curve relating the amount of glucose equivalents to absorbance ($A_{540}$) was employed for determination of enzyme activity. The formation of glucose was used as a linear function of enzyme concentration extracted from the apoplastic fluid (Fig 4.1). β-1,3-glucanase activity was expressed as mg glucose mg⁻¹ protein min⁻¹.

4.2.6 Chitinase assay
Chitinase activity was carried out with dye-labeled CM-Chitin (Loewe Biochemica GmbH, Munchen, Germany) as a substrate (Wirth and Wolf, 1990). The reaction mixture contained 100 μl of a 2 mg ml⁻¹ substrate, 20 μl enzyme and 280 μl 66.6 mM sodium acetate buffer (pH 5.0). The buffer was prepared by dissolving 0.54 g Na-acetate in 80 ml of distilled water. The pH was adjusted with glacial acetic acid and the reaction volume was made up to 100 ml. After incubation at 37°C for 30 min, the reaction was terminated by the addition of 2 M HCl, causing precipitation of the non-degrading substrate. The reaction vials were cooled on ice for 10 min and centrifuged (12 000 g for 7 min) at Mikro rapid/k (Hettich zentrifugen, Tuttlingen, Germany). Subsequently, 400 μl supernatant was transferred to a plastic cuvette and the absorbance measured at 550 nm using a Cary 3 UV-Visible spectrophotometer (Varian, Australia). The absorbance ($A_{550}$) was taken as a measure for enzyme activity, which was expressed as $A_{550}$ mg⁻¹ protein min⁻¹.

4.2.7 Peroxidase assay
The peroxidase assay was carried out as described by Zieslin and Ben-Zaken (1991) with some modifications. The reaction was based on the oxidizing of guaiacol to coloured oxidation products in the presence of $H_2O_2$ and peroxidase. The assay solution contained 1.5 ml 0.1 M phosphate buffer (pH 5.0), 10 μl apoplastic fluid and 100 μl mixture of 0.5% (v/v) hydrogen peroxide and 0.5% (v/v) guaiacol. The changes in absorbance were
Fig. 4.1. The relationship between glucose equivalents and absorbance at A₅₄₀ nm (mean of three replicates).
measured for 280 seconds at 470 nm. Peroxidase activity was expressed as μmol tetraguaiacol mg⁻¹ protein min⁻¹.

All assays were performed in triplicate and the entire experiment was repeated over time.

4.3 RESULTS
Initially, the intercellular β-1,3-glucanase activity was low for both resistant and susceptible plants (Fig. 4.2). This activity was constitutively induced in both resistant and susceptible plants from 24 hpi onwards. Until 48 hpi no significant differences in β-1,3-glucanase activity were detected between resistant and susceptible plants. After this period β-1,3-glucanase activity in both infected and uninfected resistant plants remained almost constant, while the susceptible plants showed some fluctuations. The constitutive levels of β-1,3-glucanase activity significantly increased in susceptible plants between 96 and 168 hpi and remained constant till the end of the investigation period. Stripe rust infection significantly decreased the levels of β-1,3-glucanase activity in these plants between 96 and 168 hpi, followed by a rapid increase. At 336 hpi both infected and uninfected susceptible plants displayed similar levels of β-1,3-glucanase activity.

Chitinase activity in both infected and uninfected wheat lines was low and remained relatively constant during the investigation period (Fig. 4.3).

The initial levels of peroxidase activity were low for both resistant and susceptible plants (Fig. 4.4). Within 96 hpi the activity gradually increased in both plants groups. Between 96 and 168 hpi the peroxidase activity in resistant plants continued to increase, while in susceptible plants it remained constant. Both resistant and susceptible plants had enhanced levels of peroxidase activity after 168 hpi.
Fig. 4.2. Effect of stripe rust infection on the intercellular β-1,3-glucanase of resistant Yr15/6AvS (R) and susceptible Avocet 'S' (S) wheat seedlings. Data represented are means and standard deviations from three replicates.
Fig. 4.3. Effect of stripe rust infection on the intercellular chitinase activity of resistant *Yr15/*6AvS (R) and susceptible Avocet 'S' (S) wheat seedlings. Data represented are means and standard deviations from three replicates.
Fig. 4.4: Effect of stripe rust infection on the intercellular peroxidase activity of resistant Yr15/AvS (R) and susceptible Avocet 'S' (S) wheat seedlings. Data represented are means and standard deviations from three replicates.
4.4. DISCUSSION

β-1,3-glucanase and chitinase have received considerable attention as PR-proteins having antifungal activity (Mauch et al., 1988; Ji and Kuc, 1996; Yoshikawa et al., 1990; Ham et al., 1991). Moreover, these hydrolases have been shown to play a defensive role during both early and late stages of infection, depending on the levels of constitutive expression and rapidity of induction (Joosten and De Wit, 1989; Punja and Zang, 1993; Zhu et al., 1994; Lawrence et al., 1996; Roulin et al., 1997).

The present study clearly shows an induction of β-1,3-glucanase activity within 96 hpi in both resistant and susceptible genotypes (Fig. 4.2). This induction, however, was constitutive and did not depend on the infection process. After this period activity remained almost constant in resistant plants with a slight increase at 336 hpi. Although a suppression of β-1,3-glucanase activity was noted in infected susceptible plants at 168 hpi, their response in general was similar to the resistant plants (Fig. 4.2). Likewise, no changes of chitinase activity were observed between resistant and susceptible plants (Fig. 4.3).

Although the β-1,3-glucanases and chitinases have been implicated in different plant-rust interactions (Sock et al., 1990; Munch-Garthoff et al., 1997; Hu and Rijkenberg, 1998; Anguelova et al., 1999; Kemp et al., 1999), the defensive role of these hydrolases in wheat stripe rust infection is unclear. It might be that the extracellular β-1,3-glucanases and chitinases are not involved in the resistance response of wheat to stripe rust. It has been shown that only class I vacuolar tobacco chitinases and β-1,3-glucanases have antifungal properties against Fusarium solani, while the acidic extracellular isoforms (class II) were not associated with antifungal activity (Sela-Buurlage et al., 1993). In in vitro experiments Joosten et al. (1995) demonstrated that Cladosporium fulvum is not sensitive to the acidic, extracellular chitinases and β-1,3-glucanases of its tomato host. Mauch et al. (1988) showed that
chitinases and β-1,3-glucanases present in protein extracts from pea pods infected with *Fusarium solani* act synergistically on various fungi. However, three out of 18 fungi tested were found to be insensitive to these hydrolases, while two fungal species showed weak sensitivity. These studies suggest that the involvement of host hydrolytic enzymes in the defense response varies for different host-pathogen combinations.

If β-1,3-glucanases and chitinases are not involved in the defense response of wheat to *P. striiformis*, other mechanisms of fungal arrestation are implied. In this study the resistant plants reacted hypersensitively to *P. striiformis*. As in other plant-pathogen interactions, the HR is accompanied by the generation of active oxygen species (AOS) (Mehdy, 1994). These AOS may not only mediate host cell necrosis, callose deposition and lignification (Mehdy, 1994), but might also affect the penetrating hyphae directly (Lamb and Dixon, 1997). This would indicate that the HR, together with defense responses other than β-1,3-glucanase and chitinase accumulation, are critical factors in the defense of wheat to *P. striiformis*.

A pronounced increase of peroxidase activity between 0 and 168 hpi, in both infected and uninfected resistant and susceptible plants (Fig. 4.4) was observed, suggesting a constitutive expression of this enzyme. The level of activity reached 168 hpi was two times higher in resistant plants, when compared with the susceptible plants. It has been shown that the extracellular peroxidases are involved in many events that appear early during the infection (Moerschbacher *et al*., 1988; Milosevic and Slusarenko, 1996; Lamb and Dixon, 1997). The higher level of peroxidase activity observed in *Yr15* plants compared with susceptible plants, may either ensure an early production of higher levels of *H₂O₂* which are directly antimicrobial (Peng and Kuc, 1992; Wu *et al*., 1997), or may participate in the lignification as has been shown in a case of wheat stem rust resistance (Moerschbacher *et al*., 1986; 1988), or may be involved in cell wall strengthening, which has been shown to appear within 5 min post-inoculation (Dixon *et al*., 1994).
Although the role of peroxidases in the resistance of wheat to stripe rust can not be clearly defined at present, there are indications that this enzyme may be involved in some defense reactions.
Chapter 5

MOLECULAR MARKERS FOR Yr15 RESISTANCE TO WHEAT STRIPE RUST USING AFLP TECHNOLOGY

5.1. INTRODUCTION

The first appearance of stripe rust in South Africa was recorded in 1996 (Pretorius et al., 1997). In early 1997 the disease was detected in the western Free State from where it spread throughout the rest of the country (Kloppers et al., 1997), causing significant losses in wheat production. Severe epidemics were also experienced in 1998, when a pathotype change occurred in the eastern Free State (Boshoff and Pretorius, 1999). The most economical way of controlling wheat stripe rust is through breeding for resistance. According to McIntosh et al. (1995) 18 Yr genes for resistance against stripe rust have been designated.

The gene Yr15 has been identified as a potential source of stripe rust resistance in South Africa. This gene is found on the short arm of chromosome 1B of wheat (McIntosh et al., 1996). Sun et al. (1997) identified two molecular markers, OPB13 and Nor1, flanking Yr15 by 27.1 cM and 11.0 cM, respectively. These markers were produced using Random Amplified Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphisms (RFLPs). Only one RAPD marker, out of 340 primers tested, was found to be linked to the Yr15 gene. However, in studies conducted by the Agricultural Research Council, the OPB13 primer failed to distinguish between a Yr15 tester line and control cultivars (R. Prins, ARC Small Grain Institute, Stellenbosch, personal communication, 1999).
The recently developed AFLP technique (Amplified Fragment Length Polymorphism) is proving very successful in DNA fingerprinting of cultivars and genes. This technique, although more costly per reaction, is more robust, detects more loci than RAPDs, and is becoming the standard methodology for genetic mapping.

In this study we used the AFLP technique to detect possible markers for the Yr15 gene in a South African wheat cultivar.

5.2 MATERIALS AND METHODS

5.2.1 Host genotypes
An F2 population derived from a cross between Yr15/6*AvS and the stripe rust-susceptible wheat cultivar SST55 was supplied by the Department of Plant Pathology, University of the Orange Free State. One hundred and six F2 plants were grown in a soil-peat moss mixture in 1-l-capacity pots and inoculated three weeks after planting with P. striiformis f. sp. tritici, pathotype 6E16. Following incubation of 30 h at 7°C, plants were kept at 18°C in a growth cabinet where a 14 h daylength was provided by cool-white tubes and incandescent bulbs. Plants were rated 14 days later for segregation of resistance.

DNA was extracted from each plant, prior to inoculation, as well as from the parents. To investigate possible polymorphisms between resistant and susceptible segregants, DNA from 10 resistant and 10 susceptible plants was bulked. F2 plants were grown to maturity and individually harvested. F3 families (approximately 30 plants per family) were inoculated eight days after sowing to confirm the reaction of F2 segregants, specifically those genotypes used in the molecular study.

5.2.2 DNA preparation
Wheat DNA samples were prepared with modifications according to previously described procedures (Edwards et al., 1991). An additional CTAB step was incorporated to remove proteins. This followed after the original incubation with the extraction buffer and contained 10 ml 1M Tris-HCl, 10 ml 0.25 M EDTA and 5 g CTAB. The reaction volume was adjusted to 50 ml using sterile water.

5.2.3 AFLP protocol
To generate AFLP profiles, the AFLP™ Analysis System I and AFLP Starter Primer Kit (GibcoBRL) were used with modification. The preselective amplification reaction step was extended from 1 min to 2 min while the selective amplification reaction step was also extended to 2 min.

The preselective primers were used as described by Maughan et al. (1996). For selective amplification Eco-AAC (5'-GACTGCGTACCAATTC-3') and Eco-ACA (5'-GACTGCGTACCAATTC-3') were used in all combinations with Mse-CAA (5'-GATGAGTCCTGAGTAA-3'), Mse-CAC (5'-GATGAGTCCTGAGTAA-3'), Mse-CAG (5'-GATGAGTCCTGAGTAA-3'), Mse-CAT (5'-GATGAGTCCTGAGTAA-3'), Mse-CTA (5'-GATGAGTCCTGAGTAA-3'), Mse-CTC (5'-GATGAGTCCTGAGTAA-3'), Mse-CTG (5'-GATGAGTCCTGAGTAA-3'), and Mse-CTT (5'-GATGAGTCCTGAGTAA-3'). The Eco-primers were labelled fluorescently with Fam and Ned for detection using a Perkin Elmer 310 capillary sequencer (PE Biosystems).

5.2.4 Data analysis
AFLP profiles were scored manually for the presence or absence of amplified bands to determine polymorphisms between the susceptible and resistant groups as well as the parent lines.

5.3 RESULTS
The F₂ population segregated into 96 resistant and 10 (χ²₃:₁ = 13.699) susceptible plants (Table 5.1). In this experiment infection types 0, 0⁺, ;C, ;N and ;2CN were considered to indicate resistance. Plants were rated as susceptible when infection types 3C, 3N, 3, 3+ and 4 were allocated. The parental lines SST55 and Yr15*6/AvS produced infection types 3++ and 0 to pathotype 6E16, respectively.

The segregation pattern for 105 F₃ families conformed to the ratio of 1 homozygous resistant : 2 segregating or heterozygous : 1 homozygous susceptible (χ²₁:₂:₁ = 4.359) as expected for monogenic inheritance of resistance (Table 5.1). In nine F₃ families, derived from plants regarded as resistant in the F₂, particularly those that produced the very distinct ;C or ;N reactions, susceptibility was indicated. All plants classified as susceptible in the F₂ and used for molecular analysis, reacted accordingly in the F₃. Similarly, all the resistant F₂ plants used for DNA extraction yielded either homozygous resistant or segregating F₃ progeny, confirming the presence of Yr15 in the former generation.

In the marker study only two of the primer combinations (Mse-CAA + Eco-AAC and Mse-CAA + Eco-ACA) yielded polymorphic results between the susceptible and resistant parents, as well as between the bulked susceptible and resistant F₂ DNA. Several polymorphisms between the bulked F₂ DNA were detected with these primer combinations (Tables 5.2 to 5.3). Fragment sizes ranged from 65 to 385 bp. For primer pair Mse-CAA + Eco-ACA an average of 12 fragments were detected whereof nine (65, 85, 95, 135, 140, 160, 175, 215 and 345 bp) were specific for resistant F₂ progeny and three (80, 300 and 375 bp) were only found in susceptible progeny (Table 5.2). Similarly for primer pair Mse-CAA + Eco-AAC an average of six polymorphic fragments were detected whereof three (210, 280 and 385) were specific for the resistant F₂ progeny and three (45, 300 and 375 bp) for the susceptible group (Table 5.3).
Table 5.1. Segregation ratios observed in $F_2$ and $F_3$ populations derived from a cross between SST55 and *Yr15/*6AvS. Genotypes were screened with *Puccinia striiformis* f. sp. *tritici* pathotype 6E16

<table>
<thead>
<tr>
<th>Generation</th>
<th>Number of plants or families</th>
<th>Expected ratio</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant</td>
<td>Segregating</td>
<td>Susceptible</td>
</tr>
<tr>
<td>$F_2$</td>
<td>96</td>
<td>10</td>
<td>3:1</td>
</tr>
<tr>
<td>$F_3$</td>
<td>35</td>
<td>50</td>
<td>20</td>
</tr>
</tbody>
</table>

$^a$ Deviated significantly ($P<0.005$) from expected ratio
Table 5.2. Polymorphisms observed between the bulked resistant and susceptible F₂ progeny of a cross between SST55 and Yr15/*6AvS. Values are indicative of the presence of a polymorphic fragment as well as the fragment size in base pairs. The profile was obtained with the primer combination Mse-CAA + Eco-ACA.

<table>
<thead>
<tr>
<th>Resistant F₂ progeny</th>
<th>Susceptible F₂ progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>80</td>
</tr>
<tr>
<td>85</td>
<td></td>
</tr>
<tr>
<td>95</td>
<td></td>
</tr>
<tr>
<td>135</td>
<td></td>
</tr>
<tr>
<td>140</td>
<td></td>
</tr>
<tr>
<td>160</td>
<td></td>
</tr>
<tr>
<td>175</td>
<td></td>
</tr>
<tr>
<td>215</td>
<td>300</td>
</tr>
<tr>
<td>345</td>
<td>375</td>
</tr>
</tbody>
</table>
Table 5.3. Polymorphisms observed between the bulked resistant and susceptible F$_2$ progeny of a cross between SST55 and Yr15/6AvS. Values are indicative of the presence of a polymorphic fragment as well as the fragment size in base pairs. The profile was obtained with the primer combination Mse-CAA + Eco-AAC.

<table>
<thead>
<tr>
<th>Resistant F$_2$ progeny</th>
<th>Susceptible F$_2$ progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>210</td>
<td>45</td>
</tr>
<tr>
<td>280</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td></td>
</tr>
<tr>
<td>375</td>
<td></td>
</tr>
<tr>
<td>385</td>
<td></td>
</tr>
</tbody>
</table>
Using primers Eco-ACA and Mse-CAA an average of 13 polymorphic fragments were detected between the susceptible and resistant parents whereof seven (95, 100, 285, 315, 390, 395 and 420 bp) were specific for the resistant parent and six (50, 70, 85, 295, 365 and 370 bp) were specific for the susceptible parent (Table 5.4). Similarly using primers Eco-AAC and Mse-CAA an average of nine polymorphic fragments were detected between the susceptible and resistant parents whereof seven (200, 285, 335, 350, 390, 395 and 420 bp) were specific for the resistant parent and two (150 and 295 bp) were specific for the susceptible parents (Table 5.5).

Polymorphisms between the susceptible parent (SST55), the donor parent (Yr15) and the F2 progeny are summarized in Tables 5.6 and 5.7. When comparing parental and F2 lines using primer pair Mse-CAA + Eco-ACA a total of 20 polymorphic fragments were detected whereof three (70, 115 and 295 bp) were present in SST55, eight (95, 110, 155, 285, 325, 390, 400 and 420 bp) were present in Yr15 and 10 (50, 75, 85, 95, 130, 135, 160, 205, 235 and 380 bp) were present in the F2 progeny. Of these, 6 were resistant, 3 susceptible and 1 susceptible and resistant. When comparing parental and F2 lines using primer pair Mse-CAA + Eco-AAC a total of 12 fragments were detected whereof eight (50, 155, 205, 285, 325, 340, 400 and 420 bp) were present in Yr15 and four (160, 205, 235 and 380 bp) were present in the F2 progeny.

5.4 DISCUSSION

The F2 response of the SST55/Yr15 cross deviated significantly from that expected for a single gene. Previously, Mcintosh et al. (1996) crossed a Yr15 line with a monosomic series representing the A and B genomes. Pooled results from those crosses also deviated from the expected 3 resistant to 1 susceptible ratio, suggesting preferential transmission of the Yr15 gene.
Table 5.4. Polymorphisms observed between the two parental lines *Yr15*/6AvS and SST55. Values are indicative of the presence of a polymorphic fragment as well as the fragment size in base pairs. The profile was obtained with the primer combination Mse-CAA + Eco-ACA.

<table>
<thead>
<tr>
<th>Yr15</th>
<th>SST55</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td></td>
</tr>
<tr>
<td>85</td>
<td></td>
</tr>
<tr>
<td>95</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
</tr>
<tr>
<td>285</td>
<td>295</td>
</tr>
<tr>
<td>315</td>
<td>365</td>
</tr>
<tr>
<td>390</td>
<td>370</td>
</tr>
<tr>
<td>395</td>
<td></td>
</tr>
<tr>
<td>420</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.5. Polymorphisms observed between the two parental lines Yr15/**6AvS and SST55. Values are indicative of the presence of a polymorphic fragment as well as the fragment size in base pairs. The profile was obtained with the primer combination Mse-CAA + Eco-AAC.

<table>
<thead>
<tr>
<th>Yr15</th>
<th>SST55</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td></td>
</tr>
<tr>
<td>285</td>
<td>295</td>
</tr>
<tr>
<td>335</td>
<td></td>
</tr>
<tr>
<td>350</td>
<td></td>
</tr>
<tr>
<td>390</td>
<td></td>
</tr>
<tr>
<td>395</td>
<td></td>
</tr>
<tr>
<td>420</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.6. Polymorphisms observed between the susceptible (SST55) and resistant (Yr15/*6AvS) parental lines and the F₂ progeny of a cross between SST55 and Yr15/*6AvS. Values are indicative of the presence of a polymorphic fragment as well as the fragment size in base pairs. The profile was obtained with the primer combination Mse-CAA + Eco-ACA.

<table>
<thead>
<tr>
<th>SST55</th>
<th>F₂ progeny</th>
<th>Yr15</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>(resistant and susceptible)</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>(susceptible)</td>
<td></td>
</tr>
<tr>
<td>85</td>
<td>(resistant)</td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>(resistant)</td>
<td>95</td>
</tr>
<tr>
<td>110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>115</td>
<td></td>
<td></td>
</tr>
<tr>
<td>130</td>
<td>(resistant)</td>
<td></td>
</tr>
<tr>
<td>135</td>
<td>(resistant)</td>
<td>155</td>
</tr>
<tr>
<td>160</td>
<td>(resistant)</td>
<td></td>
</tr>
<tr>
<td>205</td>
<td>(resistant)</td>
<td></td>
</tr>
<tr>
<td>235</td>
<td>(susceptible)</td>
<td>285</td>
</tr>
<tr>
<td>295</td>
<td></td>
<td>325</td>
</tr>
<tr>
<td>380</td>
<td>(susceptible)</td>
<td>390</td>
</tr>
<tr>
<td>390</td>
<td></td>
<td>400</td>
</tr>
<tr>
<td>420</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.7. Polymorphisms observed between the susceptible (SST55) and resistant (*Yr15/6AvS*) parental lines and their F$_2$ progeny. Values are indicative of the presence of a polymorphic fragment as well as the fragment size in base pairs. The profile was obtained with the primer combination Mse-CAA +Eco-AAC.

<table>
<thead>
<tr>
<th>SST55</th>
<th>F$_2$ progeny</th>
<th>Yr15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>155</td>
</tr>
<tr>
<td>160 (resistant)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>205 (resistant)</td>
<td></td>
<td>205</td>
</tr>
<tr>
<td>235 (susceptible)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>285</td>
</tr>
<tr>
<td></td>
<td></td>
<td>325</td>
</tr>
<tr>
<td></td>
<td></td>
<td>340</td>
</tr>
<tr>
<td>380 (susceptible)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
</tr>
<tr>
<td></td>
<td></td>
<td>420</td>
</tr>
</tbody>
</table>
In the present study the F3 data did not support a preferential transmission hypothesis. However, it was clear that certain plants, especially those exhibiting a fleck reaction accompanied by severe necrosis and often mosaic symptoms, were misclassified in the F2. The reason for this is not clear as several others responded with reactions typical of either of the parents. The possibility of a second gene effective against the pathotype 6E16 was not supported by the F3 data. Should the F2 ratio be recalculated based on F3 results, then single gene inheritance is confirmed \( \chi^2_{3:1} = 2.831 \). This suggests that the selection for Yr15 in segregating progeny should be directed towards plants showing an immune reaction type, rather than severe leaf necrosis or chlorosis.

Using the AFLP technique a total of 23 putative markers for Yr15 have been identified in SST55/Yr15 F2 lines. Of these putative markers 20 (50, 70, 75, 85, 95, 110, 115, 130, 135, 155, 160, 205, 235, 285, 295, 325, 380, 390, 400 and 420 bp) were identified using primers Mse-CAA + Eco-ACA, while 11 (50, 155, 160, 205, 235, 285, 325, 340, 380, 400 and 420 bp) were identified using primers Mse-CAA + Eco-AAC. Ten (155, 160, 205, 235, 285, 325, 340, 380, 400 and 420 bp) of these putative markers were identified using Mse-CAA + Eco-AAC, as well as Mse-CAA + Eco-ACA. Of these fragments one 95 bp fragment identified using primers MseCAA + Eco-ACA and one 205 bp fragment identified using primers MseCAA + Eco-AAC were detected in the resistant parent and the resistant F2 group. This indicates that these fragments lie solely within the translocated gene fragment making them ideal candidates for further investigation. The 205 bp fragment was also identified with the primers MseCAA + Eco-ACA, but only in the resistant F2 progeny and in none of the other templates used.

It is interesting to note that several fragments present in the F2 lines were not detected in the parental lines. These recombinant fragments were more noticeable between the F2 lines and the resistant parent. This observation is reminiscent of problems associated with chromosomal drag. This inter-gene
recombination results in genetic markers being ineffectual the greater the distance between the marker and the gene.

In conclusion, we have developed a SST55 wheat line with resistance to stripe rust. Using AFLP technology we have identified several putative markers for Yr15 in this background. However, further testing of the F3 and other populations involving Yr15 is required to determine the degree of linkage between the putative markers and Yr15.
Chapter 6

SUMMARY

Stripe (yellow) rust, caused by *Puccinia striiformis* Westend f. sp. *tritici*, is a major disease of bread wheat, *Triticum aestivum* L., in South Africa. The objective of this study was to evaluate the *Yr15* gene as a potential resistance source for breeding purposes.

Histological evidence was provided that *Yr15* conditions a hypersensitive reaction typical of non-durable resistance. Fluorescence, phase contrast and scanning electron microscopy complemented each other and may be used to quantify various aspects of resistance expression in the wheat stripe rust pathosystem. The frequency of stomatal penetration was extremely low and no meaningful differences in early infection structures were observed between the resistant *Yr15* and susceptible Avocet ‘S’ lines. Papillae were not involved in the resistance response to pathotype 6E16.

In studies of defense-related proteins, an induction of β-1,3-glucanase activity in both resistant and susceptible wheat genotypes was detected within 96 h post-inoculation (hpi). This induction, however, was constitutive and did not depend on the infection process. Likewise, no changes in chitinase activity were observed between resistant and susceptible plants. A pronounced increase of peroxidase activity between 0 and 168 hpi, in both infected and uninfected resistant and susceptible plants was observed, suggesting a constitutive expression of this enzyme.

Using AFLP (Amplified Fragment Length Polymorphism) technology to detect possible molecular markers for *Yr15*, two primer combinations
yielded polymorphisms between the bulked susceptible and resistant SST55 x Yr15/6*AvS F₂ DNA, as well as between the susceptible and resistant parents. Fragment sizes ranged from 50 to 420 bp. Of these, one 95 bp fragment identified using primers MseCAA + Eco-ACA and one 205 bp fragment identified using primers MseCAA + Eco-AAC were detected in the resistant parent and the resistant F₂ group. This indicates that these fragments are closely associated with Yr15 making them ideal candidates for further investigation.
Chapter 7

OPSOMMING

Streep (geel) – roes, veroorsaak deur Puccinia striiformis Westend f. sp. tritici, is 'n belangrike siekte van broodkoring, Triticum aestivum L., in Suid-Afrika. Die doelwit van hierdie studie was om die Yr15 geen as 'n potensiële weerstandsbron te evalueer vir verdere aanwending in telingsprogramme.

Histologiese bewyse is gelewer dat Yr15 'n hipersensitiewe reaksie tipies van nie-volhoubare weerstand bemiddel. Fluoressensie, fase-kontras en skandeer-elektron-mikroskopie het mekaar gekomplementeer en kan gebruik word om sekere aspekte van weerstanduitdrukking in die koringstreeproes-patosisteem te kwantifiseer. Die frekwensie van stomatale penetrasie was laag en geen beduidende verskille in vroeë-infeksiestrukture kon tussen die bestande Yr15 en vatbare Avocet’S’ lyne waargeneem word nie. Papillae was nie betrokke by die weerstandsreaksie teen patotipe 6E16 nie.

In studies van proteïne wat met verdedigingsmeganismes geassosieer word, is 'n induksie in β-1,3-glukanase-aktiwiteit in beide bestande en vatbare lyne, binne 96 h na inokulasie waargeneem. Hierdie induksie was konstitutief en is nie beïnvloed deur die infeksieproses nie. Soortgelyk het geen verskille in chitinase-aktiwiteit tussen bestande en vatbare lyne voorgekom nie. 'n Toename in konstitutiewe peroksidase-aktiwiteit tussen 0 en 168 h na inokulasie is waargeneem by geïnfekteerde en kontrole bestande en vatbare plante.
In die toepassing van AFLP ("Amplified Fragment Length Polymorphism") tegnologie om moontlike molekulêre merkers vir *Yr15* te vind, het twee priemstuk-kombinasies polimorfismes tussen SST55 x *Yr15/6*AvS F₂ DNA, onderskeidelik saamgevoeg vir bestande en vatbare segregate, aangedui. Fragmentgroottes het gewissel tussen 50 en 420 bp. Een 95 bp fragment, soos geïdentifiseer deur die priemstukke MseCAA + Eco-ACA en een 205 bp fragment, soos geïdentifiseer deur die priemstukke MseCAA + Eco-AAC het in beide die bestande ouer en die bestande F₂ groep voorgekom. Hierdie fragmente is dus nou geassosieer met *Yr15* en is ideale kandidate vir verdere ondersoek.
Chapter 8

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