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Isolation and characterization of *Diuraphis noxia* induced Sequences from wheat line PI 294994

by

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Submitted in partial fulfillment of the requirements for the degree Master of Science in the Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria

2002

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Every experiment proves something. If it doesn't prove what you want it to prove, it proves something else.

Anon.

Preface

The results presented in this thesis follow from a study, which was carried out at the Department of Genetics and the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, Pretoria, under the supervision of Prof. A-M. Oberholster (Botha) and co-supervision of Mr. E. Venter.

The results presented here are original and have not been submitted in any form to another university.

Acknowledgements

I would like to thank the following people, organizations and institutions for their contributions toward the successful completion of this thesis:

- ◊ Prof. Anna-Maria Oberholster for her exceptional guidance, support, motivation and supervision.
- ◊ Eduard Venter for his invaluable theoretical and practical input and supervision.
- ◊ Dirk Swanevelder for his research input that assisted in the completion of Chapter IV.
- ◊ The University of Pretoria and the National Research Foundation (NRF) for financial assistance.
- ◊ My parents and family for continued support, understanding and motivation.
- ◊ Oliver Dickens for his love and a shoulder to cry on.

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Summary

Infestation by the Russian wheat aphid, *Diuraphis noxia* (Mordvilko), has caused large-scale damage to small-grain crops since its introduction into South Africa in 1978 and many other countries world-wide. The extreme damage caused by *D. noxia* in wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) has resulted in a concerted effort by scientists to investigate the mechanisms and genes involved in the resistance response. This is done in an effort to understand and ultimately use the knowledge of resistance responses to produce aphid resistant crops. The aim of the present study was to use suppression subtractive hybridization (SSH) and degenerate oligonucleotide primers to amplify cDNA of differentially expressed genes and nucleotide-binding site (NBS)-containing sequences respectively. This served as a source for the identification and characterization of disease-resistance genes in the wheat line PI 294994 after infestation with the Russian wheat aphid. SSH is an effective approach for studying the genetic nature of many biological processes by identifying differentially expressed genes. The sequences that were obtained in this study did indeed show some similarity to the underlying mechanisms involved in lesion formation and disease resistance. At the amino acid level similarities were identified with polypeptides such as lymphoma receptors. At the nucleotide level similarities were identified to enzymes involved in fibrinogen lysis and the chloroplast regulatory system. A heat shock protein gene was also identified, which may possibly play a role in the induction of the resistance response during aphid infestation. The use of PCR with degenerate oligonucleotide primers, designed from the NBS region of cloned disease resistance genes, has led to the cloning of resistance sequences in various plant species. In this study oligonucleotide primers designed from conserved motifs in the NBS domain were used to clone disease resistance gene homologues from the wheat line PI 294994. The sequences obtained showed a homology to an NBS region in lettuce, which was linked to a resistance protein candidate gene. In the wheat line PI 294994 the cloned NBS sequence was expressed at varying levels in different wheat tissues, which requires further study. However, when the full-length cDNA sequence of the NBS-containing gene was sequenced, there was no significant homology to any disease resistance genes at the nucleotide level. At the amino acid level there was homology to a kinase type resistance protein. The degenerate NBS

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PCR approach was very successful in producing a number of NBS regions, which may be used for further study even if they are not necessarily involved in disease resistance. These results have aided the process of identifying novel aphid induced transcripts after infestation and has contributed to the growing base of knowledge about the underlying mechanisms involved in lesion formation and resistance responses to insects.

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Opsomming

Sedert die eerste waarneming van Russiese koringluis (*Diuraphis noxia* Mordvilko) in Suid Afrika in 1974, is gootskaalse verliese by klein-graan gewasse gerapporteer. Dit is ook die geval vir ander lande. Die ernstige skade wat deur *D. noxia* op koring (*Triticum aestivum*) en gort (*Hordeum vulgare*) veroorsaak word, het tot 'n gesamentlike poging deur wetenskaplikes gelei om die meganismes en gene betrokke by die weerstandsreaksie te ondersoek. Die poging lei tot 'n beter begrip van die meganisme en gene betrokke by die RWA weerstandsreaksie wat uiteindelik gebruik kan word om nog meer luisweerstandbiedende gewasse te kweek. Die doelwit van die huidige studie was om subtraksie-supressie-hibridisasie (SSH) en gedegenereerde oligonukleotiedvoorvoerders te gebruik om komplimentêre-DNA (cDNA) van differensieël uitgedrukte gene en nukleotiedbindingsetels- (NBS) -bevattende volgordes onderskeidelik te amplifiseer. Dit is gedoen om siekteweerstandsgene in die koringlyn PI 294994, na infestering met die Russiese koringluis, te identifiseer en te karakteriseer. SSH is 'n effektiewe nadering om die genetiese aard van baie biologiese prosesse te bestudeer deur die identifikasie van differensieël uitgedrukte gene. Die volgordes wat in hierdie studie verkry was, het wel sommige ooreenkomste met die onderliggende meganismes gewys en was dan wel funksioneel in letselformasie en siekteweerstand. Op aminosuurvlak is ooreenkomste geïdentifiseer met onder andere limfoomreseptors. Terwyl ooreenkomste met ensieme wat by fibrinogeenoplossing en die chloroplasreguleeringssisteem betrokke is, op nukleotiedvlak geïdentifiseer is. 'n Hitte-skok-proteïen is ook geïdentifiseer wat dalk 'n rol in die induksie van die weerstandsreaksie gedurende plantluisinfestering mag speel. Die gebruik van die polimerasekettingreaksie (PCR) met gedegenereerde oligonukleotiedvoorvoerders van die NBS-gebied, het tot die klonering van weerstandvolgordes in verskeie plantsoorte gelei. In dié studie is oligonukleotiedvoorvoerders vanaf gekonserveerde motiewe in die NBS-gebied ontwerp en gebruik om die siekteweerstandgeen-homoloë van die koringlyn PI 294994 te kloneer. Die volgordes wat verkry is, het homologie met 'n NBS-gebied in blaarslaai getoon wat aan 'n weerstandproteïenkandidaatgeen gekoppel is. In die koringlyn PI 294994 is die gekloneerde NBS-volgorde in variërende vlakke, in verskillende koringweefsel uitgedruk. Hierdie aspek regverdig verdere studie. Die volledige cDNA-basisvolgorde van die NBS-bevattendegeen het geen betekenisvolle

homologie aan enige siekteweerstandsgene op nukleotiedvlak getoon nie. Op aminosuurvlak was daar wel homologie met 'n kinase-tipe weerstandsproteïen. Die gedegenereerde NBS-PCR-nadering was baie suksesvol om 'n aantal NBS-bevattende fragmente te produseer, al hou dit nie noodwendig met siekteweerstand verband nie. Hierdie fragment kan vir verdere studie gebruik word. Hierdie resultate het die proses van identifikasie van nuwe plantluis-geïnduseerde transkripte na infestasie geondersteun en bygedra tot die groeiende basis van kennis oor die onderliggende meganismes wat by letselformasie en die weerstandsreaksie teen insekte betrokke is.

CHAPTER I

Introduction

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Cereal crops such as wheat, rye, barley and oats are important in human and animal nutrition throughout the world. Any decline in cereal production results in an increase in price, which severely affects economies and ultimately human lives. This is especially true in third world countries. The main factors that influence grain production are agricultural methods, drought, disease and insect pests.

The Russian wheat aphid (RWA) (*Diuraphis noxia*) (Mordvilko) originated in southern Russia, Afghanistan, Iran and countries bordering the Mediterranean (Von Wechmar, 1984; Zwer *et al.,* 1994). It was considered a pest of wheat and barley in the Caucasus in the southern Soviet Union from early in the 1900's (Walters, 1984). *Diuraphis noxia* has now become a major pest in almost all of the wheat producing countries worldwide (Dreyer and Campbell, 1987). To date the RWA has spread from western Asia, to Africa, to South America and from there to the United States and Canada (Elsidaig and Zwer, 1993). Since its introduction into the US in 1986, yield losses of as much as 60% have been reported in years of heavy infestation with losses estimated to be in excess of $ 1 billion in small grain production (Stoetzel, 1987; Haile *et al.,* 1999; Porter and Webster, 2000). As yet the RWA has not been reported in Australia.

The symptoms of RWA infestation in susceptible wheat include rolling of leaf edges, longitudinal chlorotic streaks and chloroplast degradation (Walters *et al.,* 1980; Fouché *et al.,* 1984). The aphids and these symptoms are found mainly on the upper surface of the newest growth, axils of the leaves and inside the rolled up leaves. Under severe infestations plant growth is stunted, ears become bent, necrosis sets in and eventually the plants die (Hewitt *et al.,* 1984). These symptoms are caused by the injection of a phytotoxin by the RWA while feeding (Fouché *et al.,* 1984; Rafi *et al.,* 1997). The RWA has also been identified as the vector of a number of viruses such as, the barley yellow dwarf virus, brome mosaic virus and barley stripe mosaic virus (Rybicki and Von Wechmar, 1984).

Research on the control of the RWA began in 1980 and progress has been made in reducing losses by means of chemical application (Walters *et al.,* 1980). The aphid insecticides registered for controlling other grain aphids were proven to be ineffective against the RWA (Walters, 1984). Progress has been made in developing more

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effective systemic and contact insecticides. However, due to the extremely severe effect of the RWA on wheat the application of insecticides is an expensive process. The application of insecticides also has a detrimental effect on the environment (Du Toit, 1989b). Another way of controlling the RWA is by means of biological control. During 1988 the first natural enemies of the RWA were introduced from areas where the RWA originated. Five parasites were identified with potential for controlling the RWA. These included *Diaeretiella rapae, Aphelinus asychis, A. varipes, A. colemani* and *A. matricarae* (González *et al.*, 1992). However, biological control has not proven to be as effective as originally hoped. Often parasitoids do not develop early enough to control the RWA. The predators and parasitoids also have natural enemies of their own that could limit their population numbers. The RWA is also protected from larger predators by the tightly rolled leaves of the plants on which they live and feed (Robinson, 1992). In addition, chemical spraying of fields to control other pests has a detrimental effect on the parasitoids. A RWA control program is needed that incorporates a resistant cultivar as the major control mechanism, aided by natural enemies that include predators and parasitoids and entomophagous fungi (Robinson, 1992). Thus, development of resistant wheat cultivars has become of great importance for the South African wheat industry.

Plant resistance research was initiated in 1985, when genetic resistance to the RWA was identified in certain bread wheat lines (Du Toit and Van Niekerk, 1985). In 1986, a number of sources of resistance to the RWA were found and through repeated backcrosses this resistance was successfully introduced into susceptible wheat cultivars with desirable agronomic traits (Du Toit, 1988 and 1989a). Du Toit (1987 and 1988) identified resistance to the RWA in three hexaploid wheat lines that originated in countries where the aphid is endemic. The lines PI 137739 from Iran, PI 262660 from the former USSR and PI 294994 from Bulgaria, proved highly resistant to the RWA. Later, Du Toit (1989a) found that unlinked single dominant genes conferred the resistance of PI 137739 and PI 262660. They were designated *Dn1* and *Dn2* respectively. In the PI 294994 line the visible signs of resistance are clearly evident. However, there was some speculation about the number of genes involved, as well as the mechanism of resistance (Dong and Quick, 1995). A recessive resistance gene was reported in *Triticum tauschii* and has been designated *Dn3* (Nkongolo *et al.*, 1991a). Another wheat line PI 372129 is also controlled by a dominant gene different

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from *Dn1* and *Dn2* and was assigned *Dn4* (Nkongolo *et al.*, 1991b). PI 243781 was found to have a dominant resistance gene different from all of the above genes and was assigned *Dn6* (Saidi and Quick, 1996). Today the resistance in PI 294994 is still unclear. Marais and Du Toit (1993) identified single gene inheritance and assigned it *Dn5*, but other studies have shown that two genes, one dominant and one recessive (Elsidaig and Zwer, 1993) or both dominant (Saidi and Quick, 1996) control the resistance.

Infested wheat plants are under stress and the RWA induces the hypersensitive response (HR) in resistant plants (Kindler *et al.*, 1995; Haile *et al.*, 1999). The HR is manifested through programmed cell death. Studies have shown that the RWA injects a phytotoxin into the plant while feeding, which results in typical hypersensitive response symptoms (Fouché *et al.*, 1984). The usual symptoms associated with HR are necrotic lesions and accumulation of a number of defense related products, like PR proteins (Van Der Westhuizen and Botha, 1993). Prior to infestation, protein profiles are the same for both resistant and susceptible cultivars. However, after RWA infestation there is a dramatic change in the protein profiles of the resistant cultivars (Van Der Westhuizen and Pretorius, 1995 and 1996). Porter and Webster (2000) found that feeding by the RWA inhibits synthesis and accumulation of certain proteins that are required for normal metabolic functions in susceptible plants.

Although resistant cultivars have been developed and released, the evolution of new RWA biotypes may occur to overcome this resistance. Thus, new cultivars with broader resistance against the RWA must be developed. To apply molecular techniques to modify plants with better agronomic characteristics, it is essential to understand the biochemical processes that are involved. Identification of the resistance factors in plants and the genes controlling these factors have proven useful for developing new plant varieties in tomato, maize and other cereals (Bent, 1996; Hammond-Kosack and Jones, 1996; Pointer *et al.*, 1998). The progress in identifying resistant wheat lines in South Africa and the world will assist future breeding programs for the development of resistant varieties of host plants (Du Toit, 1987, 1988; 1989a; 1989b; Nkongolo *et al.*, 1989, 1991a, 1991b).

This study is aimed at elucidating the RWA resistance gene/s in PI 294994. Suppression subtractive hybridization (SSH) was used to enrich for cDNA fragments that are differentially expressed upon RWA infestation. These induced fragments were sequenced to better our understanding of how the host plant interacts with an insect pathogen (Chapter 3). Degenerate oligonucleotide primers were also used to amplify Nucleotide Binding Site (NBS)-containing sequences for the identification of disease resistance genes. The isolated fragments were further characterized using segregating wheat populations (Chapter 4). Once disease resistance genes are identified they may then be used in the future to transfer RWA resistance to susceptible wheat lines with agronomically important traits.

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CHAPTER II

Literature Review

1. General background

1.1 Importance of wheat

Cereal cultivation was the start of civilization, as we know it. The course of human history was changed with man giving up his nomadic hunter ways and becoming a cultivator and farmer. These changes were implemented when realization dawned that the seeds from some grasses could be harvested, stored and planted again the next season. This produced more food and left enough to plant yet again. The evolution of the Gramineae plants allowed a more stable and settled life. Man was now able to provide for those unable or unlikely to survive in a hostile world, namely the young, old and disabled. More people were thus freed from basic survival to become artists, teachers, healers and philosophers (Chrispeels and Sadava, 1994).

Today wheat provides about two thirds of the total food calories for the people of the world (Dyson, 1999) and is a main staple for about 40% of the world's population (Alexandratos, 1999). With wheat playing such an important role in the feeding of so many people any loss of yield, due to pests and diseases, may represent a threat to our continued existence. Populations are growing so rapidly that it is not possible to store enough grain to sustain everyone in years of low production (Johnson, 1999). This means that grain yield stability has become exceedingly important.

1.2 Effects of disease on food security

The slightest decrease in cereal yield due to disease can cause severe cases of starvation especially in developing countries. One of the major ways that yields are stabilized is through the control of plant disease. Man has caused the disease problems of today. All cereal crops are cultivated on an ever-increasing scale, resulting in monocultures and genetically identical hosts for plant pathogens (Jones and Clifford, 1983). In an environment such as this, a parasite may take on epidemic proportions.

The beginning of the 20th century brought about a huge attempt to combat plant diseases by utilizing resistance in the host plant. When genes were discovered in the host plant that control resistance, many plant-breeding programs were initiated to include these genes into cereal crops (Ausemus *et al.*, 1967; Heyne and Smith, 1967). The success was unfortunately short-lived due to the selection pressure placed on the

pathogen. Mutants evolved that were capable of overcoming the host plant resistance (Thomas, 1999). The failure of single genes to provide sustainable resistance has led to research into the more stable polygenically controlled resistance (Rommens and Kishore, 2000). Thus, it is clear that a thorough understanding of the nature of resistance and the genes involved, is of vital importance.

2. The Russian wheat aphid (*Diuraphis noxia* Mordvilko)

2.1 Features of the Russian wheat aphid

The Russian wheat aphid (RWA) is a relatively small aphid (Fig. 2.1). It is 1.5-2 mm long, with an elongated, spindle shaped, lime green body (Du Toit and Aalbersberg, 1980; Walters *et al.*, 1980). The body may sometimes be covered with a white waxy powder (González *et al.*, 1992). The RWA is distinguished from other aphids, which are commonly found on wheat, by their extremely short antennae, since other aphids have antennae that are at least the length of the body. They have no cornicles and a unique projection above the cauda causes the characteristic forked tail appearance (Walters *et al.*, 1980; Sipes, 1997; Robinson, 1992).

Figure 2.1 The Russian wheat aphid (*Diuraphis noxia*)(Hein *et al.*, 1998).

In South Africa, only female aphids are found and reproduction occurs parthenogenetically, that is without fertilization (Walters *et al.*, 1980). The adult forms may be either winged or non-winged. The winged forms develop only when feeding conditions become unfavourable, like over crowding or when host plants are under stress. This aids in the distribution of the aphids to other plants. When the winged female finds a favourable host plant she feeds and begins to produce nymphs.

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These are identical to the adults, only smaller (Brooks and Sloderbeck, 1988). The nymphs grow into wingless adults within one week (Dreyer and Campbell, 1987) and are able to produce nymphs of their own within two weeks. The rate of production peaks at around four nymphs per day.

2.2 Origin and distribution

The RWA was first described early in the 1900s from southern Russia (Kovalev *et al.*, 1991). It is indigenous to the original cereal producing countries such as those surrounding the Mediterranean Sea, Iran and Afghanistan (Robinson, 1992; Walters *et al.*, 1980). The RWA was introduced into South Africa in 1978 and within a couple of years had reached major pest status in almost all of the country's wheat producing areas (Walters *et al.*, 1980; Walters, 1984). The RWA was first reported in Mexico in 1980 and by 1986, it had spread to the USA, where it was found in Texas (Stoetzel, 1987; Webster *et al.*, 1987). Since then the RWA has spread as far east as northwestern China (Robinson, 1992). The RWA has spread rapidly and widely from its place of origin and has colonized very different environments. Although the RWA has not yet been reported from Australia, if it is introduced, there is no reason why it will not spread as quickly as it has done in other regions of the world.

2.3 Effect of the Russian wheat aphid on plants

2.3.1 *Colonization*

The RWA densely colonize the axils on the upper surface of the leaves and the newest growth deep in the bases of the leaf sheath (Fig. 2.2)(Walters *et al.*, 1980). However, as the numbers of aphids increase the whole plant may be colonized (González *et al.*, 1992).

Wheat, barley and triticale are the preferred host plants and while rye and oats may also be infested, they are not preferred (Brooks and Sloderbeck, 1988). The RWA does not infest maize, rice or sorghum (González *et al.*, 1992). When small grains are not available the aphids survive by infesting a range of other grasses.

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2.3.2 *Feeding by the RWA*

The RWA, like other aphids, is a sheath-feeder and probes frequently in phloem tissues, which transports mainly sugar and amino acids (Ni and Quisenberry, 1997). The sucrose in the plant sap stimulates feeding and they probe until they reach this sugar in the phloem. It is thought that the aphids process these large volumes of phloem to assimilate the low concentrations of amino acids found therein (Dreyer and Campbell, 1987).

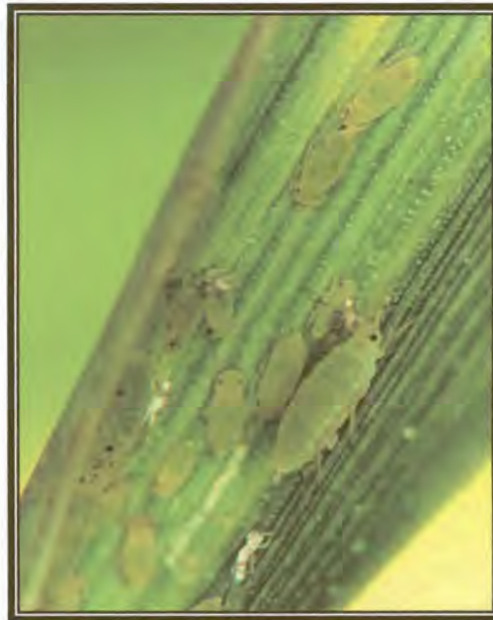

Figure 2.2 Colonization of a wheat leaf by the Russian wheat aphid (Hein *et al.*, 1998).

The RWA probes intracellularly, intercellulary or through the stomata of wheat leaf epidermal cells with a stylet. This stylet is made up of the aphid's tongue and groove-connected tubes (Miles, 1990). During probing the aphids inject saliva, which contains pectinases, into the intercellular spaces of the plant. The pectinase that is injected breaks down the pectin of the middle lamellar, as well as the cell wall pectin. This results in cell death, which can be seen as chlorosis at the site of aphid feeding (Ni and Quisenberry, 1997; McAllan and Adams, 1961). Aphid saliva may also increase phloem flow through the injection of β-1,3-glucosidase, which assists in feeding (Dreyer and Campbell, 1987). The RWA is also capable of transmitting viruses while feeding (Rybicki and Von Wechmar, 1984; Von Wechmar, 1984), which can have serious implications for the cereal industry. However, Fouché *et al.*

(1984) found that the symptoms associated with RWA feeding are caused by the injection of a phytotoxin while feeding and not by the viruses as previously conjectured.

2.3.3 *Symptoms of infestation*

Infestation and feeding of the RWA on the host plant causes a number of distinctive symptoms. A small aphid colony can cause leaf discolouration within 3-4 days (Brooks and Sloderbeck, 1988). On susceptible plants similar discolouration can be caused by as few as one aphid. The symptoms of RWA infestation on wheat are typically white, yellow and green longitudinal chlorotic streaks, that extend almost through the whole length of the leaf (Walters *et al.*, 1980). Chlorosis is caused by the destruction of the chloroplast membrane through the injection of pectinases from the aphid while it feeds (Du Toit and Aalbersberg, 1980). A reduction of the chlorophyll in infested leaves of up to 85% (Krüger and Hewitt, 1984) can reduce yield by 25-50% (Du Toit and Van Niekerk, 1985).

Infested leaves curl inwards from the edges, making the whole leaf appear tubular. The microenvironment formed by the rolled leaves partially protects the aphid from harmful effects found in the environment, like certain contact insecticides and natural enemies. This allows the aphid to feed and reproduce in safety (Robinson, 1992). As a result of the aphids being hidden, damage symptoms are often the first indication that the aphids are present. Heavily infested plants are stunted and often appear to be flattened. In more mature plants the aphids infest the spikes. The spikes become caught in the flag leaf sheath, causing them to appear white and deformed (Smith *et al.*, 1991). In extreme cases the whole plant may appear bluish-purple, which is a characteristic of plants that are under drought stress (Walters *et al.*, 1980). RWA infestation may lead to patchy patterns of dead spots in the field. When conditions are favourable, this can result in 20 to 80% infested plants within two weeks (Walters *et al.*, 1980).

2.4 Russian wheat aphid control

Considering the rapid spread and the losses involved in a RWA outbreak, a lot of research and resources has gone into identifying practices and factors that play a role

in restricting the damage done by the RWA. These include biological and chemical control of the RWA.

2.4.1 *Biological control*

The RWA arrived in many countries where there are no natural predators and parasitoids found against it. A number of expeditions have been made to countries around the aphid's site of origin to collect these natural enemies. These enemies include parasitoid wasps, beetles and predatory bugs (Robinson, 1992). This strategy has proven to be less successful than hoped because the aphids develop and emerge earlier than the predators and parasitoids and these predators and parasitoids often have natural enemies of their own. The tightly curled leaves, where the RWA live and feed, protect them from some predators, which even when in larval stage, are too large to feed within the leaves (González *et al.*, 1992). One of the advantages of using natural enemies to control the RWA, is that on resistant cultivars the resistance genes are protected by limiting the possible development of a resistance breaching biotype (Hayes, 1998). Another advantage is that once the natural enemies are present they will attack the RWA when it appears, without any further effort on the part of the farmer.

In South Africa, seven species of wasps, seven species of ladybirds and two species of flies have been found to be natural enemies of the RWA (Hayes, 1998). The wasp, *Aphelinus hordei*, was introduced as a natural parasite on RWA from the Ukraine in 1991. The female wasp lays her eggs inside the aphid and when the eggs hatch, the larva feeds on the aphid (Gonzaléz *et al.*, 1992). Other parasites (Table 2.1) used in biological control programs around the world include: *Diaeretiella rapae, Aphelinus asychus, A. varipes, Aphidius colemani* and *A. matricarae* (Sipes, 1997).

A pathogenic fungus has been identified that can control the RWA in its early stages. However, it appears to function best during the warm, wet summer and is less effective during the winter wheat growing season (Walters *et al.*, 1980). In South Africa, six species of fungi have been identified and isolated that may be of use in the biological control of the RWA (Hayes, 1998). Fungi such as *Erynia, Conidiobolus* and *Verticillium* that attack other aphids, may also be effective at controlling the

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RWA (Robinson, 1992).

Table 2.1 Natural enemies of *Diuraphis noxia*

	Name	Description	Distribution	Author
Parasites	*Aphelinus asychus*	Wasp	USA, Asia	Hayes, 1998
	Aphelinus hordei	Wasp	Asia, Africa	Prinsloo, 1998
	Aphelinus varipes	Wasp	USA, Asia	Sipes, 1997
	Aphidius colemani	Wasp	USA, Africa, Asia, Australia	Sipes, 1997
	Aphidius matricarae	Wasp	USA, Asia	Gonzaléz *et al.*, 1992
	Diaeretiella rapae	Wasp	USA, Asia, Europe	Gonzaléz *et al.*, 1992
Predators	*Adonia variegata*	Adonis' ladybird	USA, Asia	Robinson, 1992
	Chrysoperla carnea	Green lacewing fly	USA, North Africa, Russia	Tauber & Tauber, 1983
	Hippodamia convergens	Lady beetle	USA, Asia	Obrycki & Kring, 1998

2.4.2 *Chemical control*

The control of the RWA by insecticides has proven to be successful. However, most contact insecticides have insufficient penetration to where the aphid lives in the rolled up leaves. Thus, systemic insecticides are required, which have high application costs and similar to contact insecticides, adversely affect the natural enemies employed in biological control (Hayes, 1998). Another aspect causing great concern is the risk that the RWA may develop resistance to the insecticides used to control it, thereby making it more difficult to manage in the future (Robinson, 1992). These considerations, coupled with the negative effects of insecticides on the environment, have led researchers to turn to integrated control programs. This combines resistant cultivars and effective natural enemies to control the RWA.

2.5 Host plant resistance

2.5.1 *Genes conferring Russian wheat aphid resistance*

Resistant wheat cultivars are an effective, economical and environmentally friendly way of protecting crops from RWA infestation. Since RWA resistance research was initiated in 1987, thousands of wheat accessions and wheat relatives have been evaluated. To date nine genes conferring RWA resistance have been identified (Table 2.2).

Table 2.2 Russian wheat aphid resistance genes

Gene	Source	Country of Origin	Inheritance	Authors
Dn1	PI 137739	Iran	Single dominant	Du Toit, 1989
Dn2	PI 262660	Russia	Single dominant	Du Toit, 1989
Dn3	*T. tauschii* (SQ24)		Single recessive	Nkongolo *et al.*, 1991a
Dn4	PI 372129	Russia	Single dominant	Nkongolo *et al.*, 1991b
Dn5	PI 294994	Bulgaria	Single dominant 1 dominant & 1 recessive 2 dominant	Marais & Du Toit., 1993, Dong & Quick, 1995, Elsidaig & Zwer, 1993
Dn6	PI 243781	Iran	Single dominant	Saidi & Quick, 1996
Dn7	Rye	Afghanistan & Turkey	Unknown	Marais *et al.*, 1994
Dn8	PI 294994	Bulgaria	Unknown	Liu *et al.*, 2001
Dn9	PI 294994	Bulgaria	Unknown	Liu *et al.*, 2001
Dnx	PI 220127	Russia	Single dominant	Liu *et al.*, 2001

The first *Dn* (*Diuraphis noxia*) resistance genes were identified in South Africa and were designated *Dn1* and *Dn2*. These genes were present in the PI 137739 and PI

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262660 wheat accessions from Iran and Russia, respectively (Du Toit, 1987; 1988; 1989). Monosomic analysis placed the *Dn1* gene on chromosome 7D and the *Dn2* gene on chromosome 7DL (long arm) (Shroeder-Teeter *et al.*, 1994; Ma *et al.*, 1998) However, recent microsatellite studies have indicated that *Dn1* and *Dn2* are located on the short arm of chromosome 7D, near the centromere and not on 7DL (Liu *et al.*, 2001). The recessive *Dn3* gene is from the *Aegilops tauschii* line SQ24. *Aegilops tauschii* is believed to be the donor of the D genome in common wheat (Nkongolo *et al.*, 1991a). The dominant gene *Dn4* originated from the Russian bread wheat accession PI 372129 and was located on chromosome 1DS (short arm) (Nkongolo *et al.*, 1989; 1991b; Saidi and Quick, 1996; Ma *et al.*, 1998). Monosomic and diteleosomic analysis placed the *Dn5* gene, from the Bulgarian wheat accession PI 294994, on chromosome 7DL (Marais and Du Toit, 1993; Du Toit, 1988; Du Toit *et al.*, 1995). The dominant *Dn6* gene originated from the Iranian wheat accession PI 243781 and has yet to be mapped to a chromosome (Saidi and Quick, 1996; Quick *et al.*, 1991). The *Dn7* gene is derived from a rye accession but there is not a lot of information available as yet about this gene (Marais *et al.*, 1994). More recently two genes designated *Dn8* and *Dn9* have been identified. The *Dn8* and *Dn9* genes were identified using wheat microsatellites and their chromosomal locations were mapped but their mode of action has yet to be determined. These genes are also in PI 294994 where previously resistance inheritance was unclear (Liu *et al.*, 2001). The single dominant resistance gene *Dnx* in PI 220127 wheat is suspected to be a new RWA resistance gene and is located near the centromere of 7DS. Liu *et al.* (2001) found that *Dnx* is linked to but different from *Dn1*, *Dn2* and *Dn5*.

The relationships among the known *Dn* genes are complicated. Initially, *Dn1* and *Dn2* were thought to be independently inherited (Du Toit, 1989). Later, Saidi and Quick (1996) reported that *Dn1* and *Dn2* were allelic at one locus. The number and type of resistance genes in PI 294994 has also given conflicting results. Marais and Du Toit (1993) achieved results that indicated PI 294994 contained a single dominant gene and the symbol *Dn5* was assigned, they also suggested that *Dn5* and *Dn1* were linked on chromosome 7DL. At about the same time Elsidaig and Zwer (1993) found that two genes, one dominant and one recessive, conferred the resistance in PI 294994. A few years later Saidi and Quick (1996) conducted allelism tests that showed that there were three genes controlling resistance in PI 294994, two were dominant and one was

allelic to *Dn1* and *Dn2*. Another RWA resistance gene in PI 294994 may be on chromosome 1D because PI 294994 and PI 372129 (*Dn4*) share a common RWA resistance gene (Saidi and Quick, 1996) and *Dn4* is located on chromosome 1DS (Ma *et al.*, 1998). The presence of three resistance genes in PI 294994 was inferred by studies done by Zhang *et al.* (1998) with two genes on 7DL and one on 1DS. The reasons for these conflicting results may be due to the heterogeneity of the original PI 294994 wheat accession for RWA resistance genes, the effects of different parents in crosses with PI 294994 and different RWA biotypes used by the different research groups (Saidi and Quick, 1996; Zhang *et al.*, 1998).

Liu *et al.* (2001) reported that through microsatellite analysis it was determined that the *D1*, *Dn2* and *Dn5* genes are tightly linked at distances of 3.82, 3.05 and 3.20 cM, respectively. These results imply that *Dn1*, *Dn2* and *Dn5* are allelic at the same locus or are different but tightly linked to each other. They are located proximally on the short arm of chromosome 7D near the centromere and not on 7DL as previously reported. A second resistance gene in PI 294994 was located less than 3.2 cM away from the distal end of chromosome 7DS and was designated *Dn8* (Liu *et al.*, 2001). The third resistance gene was found to be located in the middle of chromosome 1DL, which is an area rich in defense genes (Boyko *et al.*, 1999). These recent results indicate that there are at least three RWA resistance genes in wheat PI 294994 and suggest relationships between *Dn1*, *Dn2* and *Dn5* (Liu *et al.*, 2001).

2.5.2 *Resistance gene mode of action*

The identification and development of host plant resistance provides an effective alternative for RWA management. However, an improved understanding of host-plant resistance requires that the modes of resistance of host plants be defined. The modes of action of these resistance genes include antibiosis, antixenosis and tolerance or a combination of the aforementioned. Antibiosis can be defined as the detrimental effect a resistant plant has on aphid biology (Kindler *et al.*, 1995). Antibiosis is typically characterized by aphids that have fed on antibiotic plants being reduced in body size, having delayed development, decreased longevity and reduced fecundity. Aphid fecundity is the most commonly assessed characteristic when determining antibiosis. It is measured as maximum nymphal production, production rate and

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length of production (Robinson, 1993; Unger and Quisenberry, 1997). Antixenosis is the measure of non-preference of the aphid for the plant. Typically this is seen as the aphids leaving the resistant plants in order to find more palatable plants (Rafi *et al.*, 1996). Tolerance is when the aphid feeds on the plant but the effect of the aphid on the plant is not accompanied by the severe symptoms of infestation such as leaf curling and chlorotic streaks (Kindler *et al.*, 1995).

The positive effects of the incorporation of the genes conferring resistance into susceptible wheat cultivars include a decrease or prevention of the formation of chlorotic streaks, the prevention of leaf rolling and lowering of nymphal production (Du Toit, 1992; Rafi *et al.*, 1996). Even though the mode of action, number and position of the genes is known, the genes themselves have not yet been fully identified and characterized.

3. Wheat (*Triticum aestivum*)

3.1 Genome composition

Wheat is a domesticated grass of the Gramineae family that originates from the dry countries of western Asia. It has been utilized as a food source since the Stone-age era about 9000 years ago (Cornell and Hoveling, 1998). The first signs that wheat was cultivated domestically are from about 7500 BC. Evidence of grain has been found in prehistoric Turkish sites, Mesopotamia (Iraq) and Syria (Kellogg, 2001). Wheat was cultivated in the UK about 2000 BC and in North America in the 15th century (Jones and Clifford, 1983).

It is thought that bread wheat was developed through an accidental hybridization between the diploid einkorn *Triticum* species and the wild grass-like *Aegilops* species and evolved through amphidiploidy. The *Triticum* species form a polyploid series based on $x = 7$ with three ploidy levels namely diploids ($2n = 2x = 14$), tetraploids ($2n = 4x = 28$) and hexaploids ($2n = 6x = 42$). They are like genomic allopolyploids because the chromosomes pair in a diploid-like way and the inheritance is disomic (Morris and Sears, 1967). The result was the hexaploid wheat *Triticum aestivum*, the bread wheat.

i 16378684
b15821146

Triticum aestivum (2n = 6x = 42) is an allopolyploid with the genome constitution AABBDD. The hexaploid wheat consists of three closely related genomes designated A, B and D. The currently held hypothesis is that the genome was formed through the hybridization of *Triticum urartu* (AA) with *Aegilops speltoides* (BB) producing the tetraploid *Triticum turgidum* (AABB). Later this tetraploid hybridized with *Triticum tauschii* (*A. squarrosa)* (DD) to form the hexaploid *Triticum aestivum* (AABBDD). This is estimated to have happened only about 8000 years ago (Devos and Gale, 1997). Unlike the smaller genome size of 130-140 MB in the model experimental plant, *Arabidopsis thaliana* or 400 MB for the model cereal, rice, wheat has a large genome estimated at between 16 000-17 000 MB. This makes it a really difficult cereal to study at the genomic level (Devos and Gale, 1997; Lagudah *et al.*, 2001).

4. Resistance response

When a plant is attacked by a pathogen it will induce a number of mechanisms to stem the invasion. The first response to be initiated after pathogen attack is the hypersensitive response (HR). A few hours later a response can be detected in the plant at a distance away from the site of invasion, this is known as the systemic acquired resistance (SAR). Since the properties and molecular interaction between the RWA and the host plant has not yet been elucidated, it is assumed the aphid-wheat interaction will show similarities with other better-defined plant-pathogen interactions.

4.1 The Hypersensitive Response (HR)

HR is the rapid localized death of cells at the site of attempted pathogen invasion (Dangl, 1995). A large number of pathogens and pests can cause the induction of the HR. HR covers the time from the death of one cell to the time a necrotic lesion forms. This lesion prevents the pathogen from forming a colony (Dangl *et al.*, 1996). This process of cell death actively provides resistance for the rest of the plant by sacrificing the infected or nearby cells, thus restricting the pathogen to small areas around the originally infected cells (Dangl, 1995). Programmed cell death (PCD) in plants is similar to apoptosis in animals and is supported by molecular evidence of conserved mechanisms in both plants and animals (Greenberg, 1997; Jabs, 1999). For pathogens

that require nutrients or the host's replication mechanisms, the death of the plant cell would effectively inhibit invasion of other cells and multiplication. For pathogens that can use nutrients from dead plant cells, the cell death may not be as effective. However, with the breakdown of the dead cell, toxic substances are released from the vacuole. Phytoalexin concentrations would also increase to prohibitive levels, as the cell no longer metabolizes them. This creates an unattractive environment for the invading pathogen (Hammond-Kosack and Jones, 1996).

The HR is initiated by the recognition of the plant for the pathogen. This is facilitated by the plant resistance (*R*) genes and the pathogen's avirulence (*avr*) genes in a gene-for-gene interaction. In the last few years several *avr* and *R* genes have been identified, cloned and studied. Programmed cell death (PCD) is a pathway with a cascade of events induced by pathogen recognition. Evidence for this is provided by the fact that the HR needs an active plant metabolism, there are signal molecules present that induce a defense response and genes are expressed that activate a HR-like PCD. Thus, HR cell death is not caused by the pathogen invasion but rather by the activation of the plant's genetic response (Jabs, 1999). HR is associated with an oxidative burst, membrane damage, ion fluxes, endonuclease activation, DNA cleavage, plant cell wall modifications and pathogenesis-related (PR) protein expression, such as chitinase and β-1,3-glucanase (Lamb, 1994; Jackson and Taylor, 1996; Heath, 1998).

The first PR proteins identified were large numbers of extracellular proteins induced as a result of a tobacco mosaic virus (TMV) infection in tobacco plants (Whitham *et al.*, 1994). Later, PR gene expression was found in many incompatible plant-pathogen interactions in *Arabidopsis*, tomato, maize and other cereals (Bent, 1996; Hammond-Kosack and Jones, 1996; Pointer *et al.*, 1998). Further study has indicated that when two or more PR proteins are expressed, there is an increase in the amount of disease control. This implies that a few PR proteins are needed to work together to confer resistance (Gilchrist, 1998). PR proteins that are found in the vacuole are probably not involved in the initial defense response but rather play a role when the cell has undergone degradation. However, the genes that encode the PR proteins found in the cytoplasm are activated soon after infection is detected and therefore are directly involved in the HR (Mittler and Lam, 1995; Hammond-Kosack and Jones, 1996).

4.2 Genes involved in the Hypersensitive Response

The resistance response depends on the plant recognizing the invading pathogen. A number of genes have been found that control this recognition, these genes are called resistance (*R*) genes. In plants there are a large number of *R* genes that are specific for certain pathogens. Although different plants react differently to different pathogens, there are some similarities in *R* gene mediated defense responses (Bent, 1996). A pathogen has an *avr* gene, which if it is expressed, produces a signal that is recognized by the product of the specific *R* gene in the plant. The *R* gene in turn induces the resistance response. This is known as gene-for-gene resistance (Keen, 1992; Staskawicz *et al.,* 1995). However, if the plant does not have the *R* gene specific for the pathogen, the pathogen will successfully invade the plant. One plant may have a large number of *R* genes and a pathogen may have a large number of *avr* genes. The strongest resistance occurs when the *R* gene that is expressed matches the *avr* gene (Stahl and Bishop, 2000).

More than 10 years ago the first *avr* gene was cloned from a pathogen and since then a large number of *avr* genes have been identified (Keen, 1990). Even though the *avr* genes were cloned and sequenced, neither their functions in the pathogen nor the way in which they activate the HR was revealed. The currently held hypothesis is that the protein produced from the *avr* gene of the pathogen acts as a recognition factor by the *R* gene product, resulting in a defense response (Rommens and Kishore, 2000). The *avr* genes of the pathogen routinely undergo changes caused by gene rearrangements, insertions and deletions. The advantage of these changes is that new *avr* gene alleles are formed that overcome the existing disease resistance. The *R* genes must therefor also have a mechanism to overcome new pathogen infections (Greenberg *et al.*, 1994).

4.3 Systemic Acquired Resistance (SAR)

SAR is a signal transduction pathway that is involved in providing durable protection for the plant from pathogens. SAR is initiated during the HR when a lesion is formed and this results in disease resistance that is present through the plant's entire system (Ryals *et al.*, 1996). SAR is like a quantitative resistance response to a pathogen because it protects against a wide range of pathogens and is characterized by differences in gene expression. SAR appears to enhance other disease resistance

responses, since it can change compatible plant-pathogen interactions (i.e. disease causing) into incompatible interactions (i.e. non-disease causing)(Dangl *et al.*, 1996).

SAR was first identified as a resistance response to pathogens almost 100 years ago (Ryals *et al.*, 1996). However, it was only recently that SAR genes were identified in uninfected regions of the plant (Dangl *et al.*, 1996). SAR proteins are those proteins that are found or activated during the SAR response to maintain resistance to the pathogen. SAR proteins were analyzed and it was found that they are related to the PR proteins (Greenberg, 1997). The genes that encode these proteins have been cloned, characterized and used to determine when SAR is induced (Levine *et al.*, 1996). This information resulted in the confirmation that the SAR only occurs when the resistance response is initiated during pathogen invasion. Thus, SAR does not occur in all cases of cell death such as during senescence, wounding or freezing (Jabs, 1999).

4.4 Gene sequences related to defense

In the late 1980's, several laboratories initiated experiments to isolate different *R* genes. One of the most remarkable features discovered about the *R* genes in many different plant species is that it encodes proteins that have strikingly similar structures. The implication is that the defense response process is conserved between a wide variety of plant species (Bent, 1996). Sequence analysis has lead to the identification of a number of structural motifs found in resistance gene products. The structural domains found are serine-threonine kinases, leucine-rich repeats, nucleotide-binding sites, leucine zippers, Toll/interleukin-1 resistance domains and transmembrane receptor kinases (Rommens *et al.*, 1995; Bent, 1996; Jackson and Taylor, 1996; Keen, 1992).

4.4.1 *Serine-Threonine Kinases*

Pto of tomato was the first *avr* gene-specific *R* gene to be isolated (Martin *et al.*, 1993). This is the *R* gene that confers resistance against *Pseudomonas syringae* pv. *tomato* bacteria expressing the *avr* gene, *avrPto*. The cloning and characterization of *Pto* suggests that protein kinase-mediated signal transduction plays a crucial role in gene-for-gene plant disease resistance. One of the most common methods for plants to control certain protein activities is by changes in phosphorylation (Bent, 1996). A

number of protein kinases with conserved areas, have been identified that phosphorylate the serine-threonine regions (Ritter and Dangl, 1996). The amino acid sequence of the *Pto* gene has these conserved domains and has been shown to have protein kinase catalytic activity *in vitro* (Rommens *et al.*, 1995).

4.4.2 *Leucine-Rich Repeats (LRR)*

LRRs are a series of short repeats of about 24 amino acids, with leucines or other hydrophobic amino acids spaced at frequent intervals along the repeat. These leucines cause the protein to fold up into a particular tertiary structure, like a helix or a fist, with each LRR forming a single coil or finger (Bent, 1996; Jones and Jones, 1997). LRR regions in proteins are thought to assist in protein-protein and receptor-ligand interactions in many different organisms. Genetic evidence indicates that the β-strand/β-turn of the LRR, with the consensus sequence xxLxLxx, is a key region in the *R* protein and may serve as the binding domain for *avr* gene products (Jones and Jones, 1997). On the other hand, LRRs could be involved in the interaction of *R* gene products with other proteins that are involved in defense signal transduction (Jackson and Taylor, 1996). *R* gene sequence comparisons show that the x-residues in this region are hypervariable and have high ratios of non-synonymous to synonymous nucleotide substitution. The implication is that the xxLxLxx region creates a surface that has evolved to detect variations in the many pathogen ligands (Jones and Jones, 1997; Jackson and Taylor, 1996).

4.4.3 *Nucleotide-Binding Sites (NBS)*

Nucleotide binding site regions are found in a wide variety of proteins and they have ATP or GTP binding activity (Jackson and Taylor, 1996). A number of resistance genes that produce LRRs also produce sequences of amino acids that are very like NBS. Thus, they could bind ATP or GTP and then activate the defense response. Since the NBS regions are very conserved in a number of *R* gene products, it has been suggested that the binding of nucleotide triphosphates are needed for the proteins to be fully functional (Bent, 1996). Changing important sites in the NBS using site-specific mutation, has caused the inhibition of the HR in transformed plants (Keen, 1990; Bent, 1996). Some recent models view the central NBS as an adaptor region, linking the C-terminal LRR recognition domain to various N-terminal effectors

(Rommens and Kishore, 2000).

4.4.4 *Leucine Zippers (LZ)*

Within the NBS-LRR subclass of *R* genes, some *R* proteins possess a leucine zipper or coiled-coil sequence between the N-terminal and the NBS and LRR domains (Rommens and Kishore, 2000). Leucine zippers have a consensus sequence of xxxYxxL where Y represents a hydrophobic residue. In other proteins, these repeat sequences facilitate protein-protein interactions by promoting the formation of coiled-coil structures (Bent, 1996). Leucine zippers are well known for their role in homo- and heterodimerization of eukaryotic transcription factors but similar domains also cause interactions between proteins with many other functions (Stahl and Bishop, 2000).

4.4.5 *Toll/Interleukin-1 Receptor Similarity*

Other NBS-LRR *R* proteins contain a large N-terminal domain called the Toll/interleukin-1 resistance domain (TIR). This domain has some similarity to the cytoplasmic signalling domains of the *Drosophila* Toll protein, the mammalian interleukin receptor (IL-1R) and a family of mammalian Toll-like receptors, one of which participates in recognition and response to lipopolysaccharides (LPS) (Whitham *et al.*, 1994). Toll, IL-1R and the mammalian Toll homologue all contribute to the immune response (Jackson and Taylor, 1996). The presence of the TIR domain in several *R* plant proteins suggests a role for this domain in signalling but not in ligand binding (Bent, 1996).

4.4.6 *Transmembrane Receptor Kinases*

The last class of *R* genes shows the close relationship between *R* genes that encode LRR proteins and those that encode protein kinases (Jones and Jones, 1997). In this class, both of these functional domains are encoded in the same protein. *Xa21*, the rice *R* gene providing resistance against the bacterium *Xanthomonas oryzae* pv. *oryzae*, is the only member of this class at present (Bent, 1996). *Xa21* encodes a LRR receptor kinase in which the N-terminal LRRs are extracellular. The extracellular LRR is joined by a transmembrane region to a protein kinase region that is located in the cytoplasm (Bent, 1996).

5. Techniques to study disease resistance/ genome analysis

Plant breeding aims to improve agronomically relevant or interesting traits. This is achieved by combining characteristics found in different parental lines of cultivated species or their wild relatives. Conventional breeding programs attain this goal by generating a F_2 population and screening the phenotypes of the plants for the presence of the desirable trait. The next process is the time-consuming and costly procedures of repeated backcrosses, selfing and testing. The breeder must rely on screening methods and easy to identify phenotypical characteristics. Thus, the combination of quantitative trait loci, recessive genes or the pyramiding of genes encoding the same trait is difficult to achieve with classical breeding techniques. However, various molecular approaches are greatly increasing our ability to characterize and manipulate disease resistance genes in plants.

5.1 cDNA libraries and Expressed Sequence Tags (ESTs)

The rapid progress in genomics and the improvement in automated genetic analysis instruments have enabled the large-scale isolation and partial sequencing of many complementary DNA (cDNA) clones (Park *et al*., 1993). The potential value of partial cDNA sequence collections was first realized in the plant model species *Arabidopsis thaliana* (Newman *et al*., 1994). Since then several cDNA projects on rice, yeast, mouse and human genomes have been initiated (Yamamoto and Sasaki, 1997).

It is often possible to derive the putative function of an unknown gene, based on partial nucleotide or deduced amino acid sequence homology, to genes or gene products of known function (Adams *et al*., 1991). Frequently, long regions of sequence homology are identified in functionally related gene products from phylogenetically distant organisms like bacteria and humans (Adams *et al*., 1991). These partial cDNA sequences are referred to as expressed sequence tags (ESTs).

EST production is a rapid and efficient method for sampling a genome for active gene sequences. These sequences are used as tags to search existing databases to determine if a specific gene or gene motif has been found in the same or other organisms and if its function has been determined (Adams *et al*., 1995). Most of the ESTs available for wheat, are from tissues that include the developing grain, leaf, root, stress induced and

uninduced seedlings (Lagudah *et al.*, 2001). Gene function determination is still the biggest challenge in wheat genomics. Firstly, the homology of wheat ESTs with similar genes in other plants is determined. If a function is known for the gene in the other plant, then a similar function is suggested for the wheat EST (Lagudah *et al.*, 2001). Large-scale applications of EST sequencing have revealed not only the potential but also the limitations of this procedure. The limitations are imposed mainly by the fact that the majority of every organism's genes are not all represented in any given library. Usually the highly expressed genes are represented many times. However, this repetition can be limited by using normalized libraries where the highly expressed genes are reduced by subtractive hybridization (Rounsley *et al.*, 1996).

5.2 Suppression Subtractive Hybridization (SSH)

Subtractive cDNA hybridization has been an effective tool to identify and isolate cDNAs of differentially expressed genes. A number of cDNA subtraction methods have been developed and they all involve hybridization of the cDNA from a tester population to an excess of cDNA from a driver population. The differentially expressed cDNAs, the target, are present in the tester cDNA but are absent from the driver cDNA. This is followed by separation of the unhybridized target from the hybridized common sequences. However, these methods are often not able to obtain low abundance transcripts, they require large mRNA concentrations and they require many subtraction steps (Diatchenko *et al.*, 1996).

Suppression subtractive hybridization (SSH) was developed by Diatchenko *et al.* (1996), to overcome problems associated with subtraction libraries. This method is a PCR-based technique that can amplify selected differentially expressed target cDNA fragments and at the same time suppress common non-target cDNA amplification. The suppression PCR effect is caused by long inverted terminal repeats, which can selectively suppress the amplification of unwanted sequences in the PCR process when they become attached to the cDNA (Siebert *et al.*, 1995). In all PCR cycles, exponential amplification can only occur with the differentially expressed tester sequences that have two different adapters. Molecules with the same adapters on both ends form stable panhandle structures and cannot act as template for PCR, which constitutes the suppression PCR effect. Molecules with no adapters are missing

primer sites and thus no amplification will occur. Molecules with only one adapter will be amplified at a linear rate. The subtraction technique solves the problem of mRNA abundance differences by including a hybridization step that normalizes the sequence abundance during subtraction (Diatchenko *et al.*, 1996). The advantages of this method are that there is no need for the physical separation of single stranded and double stranded cDNA, only one subtraction hybridization is needed and more than a thousand-fold enrichment for rare differentially expressed cDNAs can be achieved. The disadvantage of the SSH technique is that a few micrograms of mRNA from the driver population is required, which in some cases may not always be possible to obtain (Diatchenko *et al.*, 1996).

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CHAPTER III

Isolation and Characterization of cDNA Sequences from PI 294994 wheat line (*Dn5*) after Russian wheat aphid infestation using Suppression Subtractive Hybridization

1. Introduction

Plants are continually being exposed to pathogen attack. Unlike animals, plants do not have a circulatory system and antibodies, instead they have evolved a complex defense mechanism to defend themselves. These mechanisms against pathogens divide into two classes: those that are present constitutively and those that are induced upon exposure to a pathogen (Staskawicz, 2001). Induced plant resistance to pathogens involves a complex array of biochemical and structural alterations in the plant cell. Among the cellular events that characterize resistance are the oxidative burst, cell wall strengthening, induction of defense gene expression and rapid cell death at the site of infection (Gatehouse and Gatehouse, 1998). Induced expression of a large number of defense-related genes is essential for plants to counter pathogen infections. Many defense-related genes encode proteins possessing antifungal or antibacterial activities, or enzymes that catalyze secondary metabolites linked to a defense response. Others encode regulatory proteins important for defense signal transductions (Xiao *et al.*, 2001). The isolation and characterization of these genes are essential for our understanding of plant disease resistance mechanisms.

Wheat (*Triticum aestivum* L.) is an economically important crop around the world. Its genome has been well studied in relation to the mapping of various resistance (*R*) genes and other agronomic traits (Feuillet *et al.*, 1995; Leister *et al.*, 1998; Röder *et al.*, 1998). Until 1992, no *R* gene from plants had been cloned and characterized at the molecular level (Staskawicz *et al.*, 1995). Today, *R* genes from several plant species have been cloned, including *Arabidopsis*, tomato and potato (Bent *et al.*, 1994; Rommens *et al.*, 1995; Wu *et al.*, 1995). However, the cloning of *R* genes from wheat has taken a considerably longer time due to its more complex and larger genome compared to other crops studied (Leister *et al.*, 1998; Seah *et al.*, 1998). Nevertheless, the benefits to be gained from the cloning of specific *R* genes, which are then used as co-segregating markers in selection and as intergenic sources of transgenes in transformation breeding, justifies continued effort (Assad *et al.*, 1999).

Since Russian wheat aphid (RWA) resistance research was initiated in 1987, thousands of wheat accessions and wheat relatives have been evaluated. To date nine genes conferring RWA resistance have been identified. The first *Dn* (*Diuraphis*

noxia) resistance genes, *Dn1* and *Dn2*, were identified in South Africa in the PI 137739 and PI 262660 wheat accessions, from Iran and Russia respectively (Du Toit, 1987; 1988; 1989). Both *Dn1* and *Dn2* are found on chromosome 7DS, near the centromere (Liu *et al*., 2001). A recessive resistance gene, present in *Triticum tauschii* line SQ24, was designated *Dn3* (Nkongolo *et al*., 1991a). Two non-allelic dominant resistance genes, found in PI 372129 and PI 294994, were designated *Dn4* and *Dn5* (Nkongolo *et al*., 1991b; Marais and Du Toit, 1993). The Bulgarian wheat line PI 294994, contains the *Dn5* resistance gene. The *Dn5* gene is located on chromosome 7DS, which is an area rich in defense genes near the *Dn1* and *Dn2* genes (Marais and Du Toit, 1993; Du Toit, 1988; Du Toit *et al*., 1995; Venter and Botha, 2000). The latest development in the elucidation of the RWA resistance genes in wheat PI 294994 is that there are at least three separate resistance genes that make up *Dn5* resistance (Liu *et al*., 2001). The dominant *Dn6* gene, from PI 243781, has yet to be mapped to a chromosome (Saidi and Quick, 1996). The *Dn7* gene is derived from a rye accession (Marais *et al*., 1994). Recently two genes, designated *Dn8* and *Dn9*, were identified using wheat microsatellites in the wheat line PI 294994 (Liu *et al*., 2001).

In this chapter suppression subtractive hybridization (SSH) was employed to isolate wheat cDNA corresponding to Russian wheat aphid induced transcripts after infestation. This was done to study the underlying mechanisms for cell death during lesion formation and disease resistance in the wheat line PI 294994.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

All the chemicals used were of analytical grade.

2.1.2 Plant Material

Resistant wheat seed, PI 294994 also named "Strelinskaja Mestnaja", is a hard winter wheat that originated in Hungary but was acquired from CIMMYT (Mexico). The plants were grown in pots under greenhouse conditions, with prevailing day and night cycles, at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. The temperature was maintained at about 24°C and the plants were watered daily. Half of the wheat seedlings were infested with RWA at the 3-4-leaf growth stage with about 10 aphids per plant. After a week, leaves from the uninfested plants and all heavily infested leaves were removed. The aphids were removed from the infested leaves under running water to prevent aphid derived nucleic acid contamination during the RNA isolation. The leaves were dried and used immediately for total RNA isolation.

2.1.3 Treatment of glassware, plastic ware and solutions

All glassware was treated overnight in 0.1% (v/v) diethyl pyrocarbonate (DEPC), autoclaved for 20 min at 121°C and baked at 200°C for 3-4 hours (Sambrook *et al.*, 1989). The mortars and pestles were washed in 0.25M HCL for 30 min, prior to DEPC treatment, autoclaving and baking. All plastic ware and solutions, except those containing Tris (2-Amino-2-(hydroxymethyl)-1,3-propandiol), were DEPC treated and autoclaved.

2.2 Methods

2.2.1 Total RNA Isolation

Total cellular RNA was extracted by a modified acid guanidium thiocyanate-phenol-chloroform extraction, described by Chomczynski and Sacchi (1987). All procedures were executed at 0-4°C. The infested and uninfested wheat leaves were separately

ground to a powder in liquid nitrogen with a mortar and pestle. Extraction buffer (4M guanidine thiocyanate, 25mM sodium citrate, 0.5% (w/v) N-lauroylsarcosine, 100mM 2-mercaptoethanol) was added to the homogenate in a 3:1 volume to tissue ratio. Successively, 2M sodium acetate (pH 8.00), Tris-EDTA (pH 8.00) equilibrated phenol and chloroform: acetic acid (49:1) was added and vortexed after each addition. The samples were incubated for 15 min on ice and centrifuged at 10 000xg for 20 min. An equal volume of isopropanol was added to the supernatant and the nucleic acids precipitated for 1.5-5 hours at -20°C. The samples were centrifuged at 10 000xg for 20 min and the pellet was resuspended in extraction buffer and precipitated with an equal volume of isopropanol at -20°C for 1 hour. The centrifugation was repeated as described and the pellet washed 3 times with ice-cold 75% (v/v) ethanol. The pellet was resuspended in 70% ethanol, centrifuged, dried under vacuum and dissolved in RNase free water. The RNA samples were stored at -80°C for further use.

2.2.2 RNA Concentration Determination

The RNA concentration was determined on a Beckman DU®-64 spectrophotometer, by reading the absorbance at 260nm and calculated using:

$$OD_{260} \times 40\mu g.ml^{-1} \times \text{dilution factor} = \text{concentration RNA } (\mu g.ml^{-1})$$

The 260/280 ratios were determined to indicate the level of protein contamination (Sambrook *et al.*, 1989).

2.2.3 Agarose gel electrophoresis analysis of RNA isolation

The total RNA from both samples were run on a 2 % agarose gel to determine their integrity (Sambrook *et al.*, 1989). The system was run in 1x TAE electrophoresis buffer (0.08M Tris-acetate, 0.002M EDTA pH 8.00) and the samples mixed with 6x loading buffer (15% Ficoll, 0.25% bromophenol blue) prior to loading of the gel. The molecular mass standard used was Marker III, which is λ DNA digested with *Eco*RI and *Hin*dIII (Sambrook *et al.*, 1989). The RNA isolation products were electrophoresed at 100 V for 30 min and visualized under UV light with ethidium bromide (EtBr) staining (Sambrook *et al.*, 1989).

2.2.4 mRNA Isolation and cDNA Synthesis

Messenger RNA was purified from the total RNA using Oligo(dT) Cellulose affinity

chromatography (GibcoBRL, Life Technologies). A column of 1g of oligo(dT) cellulose was poured in a Pasteur pipette, plugged with sterile glass wool. The total RNA was dissolved in TE buffer (10mM Tris-HCl pH 7.4, 1mM EDTA). The RNA was heat denatured at 65°C for 5 min. The total RNA was applied to the column and run through under gravity and then the columns were centrifuged at 350xg for 2 min. A high salt buffer (10mM Tris-HCl pH 7.40; 1mM EDTA, 0.5M NaCl) was added and centrifuged again for 2 min. The column was washed three times with a low salt buffer (10mM Tris-HCl pH 7.40; 1mM EDTA, 0.1M NaCl) and the mRNA was eluted with four volumes of elution buffer (10mM Tris-HCl pH 7.40; 1mM EDTA) that was pre-warmed to 65°C. The eluate was collected by centrifugation for 2 min. The synthesis of cDNA was carried out using the Roche Molecular Biochemicals cDNA Synthesis System according to the manufacturer's specifications. Both the uninfested and the infested wheat mRNA was used as the substrate for the cDNA synthesis reaction. The double stranded cDNA was purified by the QIAquick Spin Purification Procedure (Qiagen). The cDNA was eluted in water, the concentration determined spectrophotometrically and stored at -20°C.

2.2.4 Suppression Subtractive Hybridization (SSH)

The SSH technique used was a modified method of that described by Diatchenko *et al.*(1996). SSH was performed using both infested and uninfested cDNA samples. The cDNA from the infested wheat was designated the tester, and from the uninfested cDNA as the driver.

2.2.4.1 RsaI Digestion

The cDNA samples were digested with 15U *Rsa*I (Roche) at 37°C for 3 hours and purified by QIAquick Spin Purification (Qiagen). To determine if the samples were completely digested, undigested ds cDNA and *Rsa*I-digested cDNA were electrophoresed on a 1% agarose/EtBr gel in 1X TAE buffer.

2.2.4.2 Adapter Ligation

Adapters were ligated only to the tester cDNA from the infested individuals. The digested tester cDNA is diluted five times in water and divided into two portions. Half

the diluted tester cDNA was ligated to 10μM Adapter I (5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3', 3'-GGCCCGTCCA-5') and the other half to 10μM Adapter II (5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3', 3'-GCCGGCTCCA-5'), with 5U T4 DNA ligase (Roche), at 4°C overnight. To stop the ligation reaction 0.2M EDTA (pH 8.40) was added and the samples heated to 70°C for 5 min and stored at -20°C.

2.2.4.3 Ligation efficiency analysis

To verify that at least 25% of the cDNA has adapters on both ends, a PCR reaction was done that amplify fragments that span the adapter/cDNA junctions of both tester samples. A sample from both the ligation samples were diluted 1:100 in the same tube. Of this dilution 1μl was added to a 25μl PCR reaction containing, 10μM PCR primer P1 (5'-CTAATACGACTCACTATAGGGC-3'), 10μM PCR primer P2 (5'-TGTAGCGTGAAGACGACAGAA-3'), 1x PCR buffer, 10μM dNTPs, 2mM $MgCl_2$, and 1U Taq DNA polymerase (Promega). A PCR was also conducted using nested PCR primers, PN1 (5'-TCGAGCGGCCGCCCGGGCAGGT-3') and PN2 (5'-AGCGTGGTCGCGGCCGAGGT-3'). The PCR was performed with the following parameters: an initial cycle of 72°C for 5 min; 94°C for 30 sec; followed by 25 cycles consisting of 94°C for 10 sec; 55°C for 30 sec; 72°C for 2.5 min; with a final extension at 72°C for 7 min on a Perkin-Elmer GeneAmp PCR System 9700 (Applied Biosystems). The PCR products were analyzed on a 2% agarose/EtBr gel run in 1X TAE buffer.

2.2.4.4 First and second hybridization

An excess of driver cDNA (≈600ng) was added to each adapter 1-ligated and adapter 2-ligated tester cDNA (≈20ng), ethanol precipitated and resuspended in 1.5μl hybridization buffer [50mM Hepes (pH 8.30), 0.5M NaCl, 0.02mM EDTA , 10% (w/v) PEG 8000]. The samples were overlaid with mineral oil, heat denatured at 98°C for 1.5 min and allowed to anneal at 68°C for 10 hours in a thermal cycler. Immediately after the first hybridization, the two samples were mixed together in the presence of fresh denatured driver cDNA (≈150ng) in 1.5μl hybridization buffer. The sample was then allowed to hybridize for another 10 hours at 68°C. The final

hybridization sample was diluted in 200µl dilution buffer [20mM Hepes (pH 8.30), 50mM NaCl, 0.2mM EDTA pH 8.30), heated at 72°C for 7 min and stored at -20°C.

2.2.4.5 PCR Amplification

Differentially expressed cDNAs were selectively amplified in two PCR reactions. The primary PCR was conducted in 25µl. It consisted of 10µl diluted, subtracted cDNA, 10µM primer P1, 10µM primer P2 , 1x PCR buffer, 10µM dNTPs, 2mM $MgCl_2$ and 1U Taq DNA polymerase (Promega). Before thermal cycling, missing strands of the adapters were filled in by incubation at 75°C for 5 min. The PCR cycle was commenced immediately with the following parameters; 27 cycles at 91°C for 30 sec, 54°C for 30 sec, 72°C for 2.5 min and a final extension at 68°C for 7 min. A secondary PCR was performed using 1µl of the primary PCR product. The same conditions were used as for the primary PCR except that nested primers PN1 and PN2 replaced primers P1 and P2 respectively, 30 cycles were used instead of 27 and the annealing temperature was 64°C. The primary and secondary PCR products were analyzed by 2% agarose/EtBr gel electrophoresis run in 1x TAE buffer.

2.2.4.6 Cloning and analysis of the subtracted cDNA

The SSH fragment products from the secondary PCR were purified from the agarose gel using the QIAquick Gel Extraction kit (Qiagen). These fragments were cloned into the pGEM®-T Easy vector system (Promega) as per manufacturer's instructions. Ligation mixtures were used to transform competent *E. coli* (JM109) cells. Plasmid DNA was isolated from candidate clones and purified. Sense and antisense strands of the clones were used in cycle sequencing with the BigDye Terminator Cycle Sequencing Reaction kit (Perkin-Elmer). The products were separated on the ABI-3100 Prism Automated sequencer (Perkin-Elmer). The sequences were used in BLAST searches and they were aligned to other published sequences in GenBank (Altschul *et al.*, 1997).

3. Results

3.1 Suppression Subtractive Hybridization

3.1.1 RNA Isolation and cDNA Synthesis

Total RNA was isolated from the infested and uninfested wheat PI 294994 (Fig. 3.1). The RNA samples were enriched for the mRNA fraction and double stranded (ds) cDNA was synthesized. The cDNA that was synthesized from the infested (induced) individuals was designated as the "tester" and that from the uninfested (uninduced) individuals as the "driver". The cDNA derived from the mRNA appears as a smear from 0.5-3 kb. After *Rsa*I digestion, the average cDNA size was smaller resulting in a smear from 0.1-2 kb (Fig. 3.2). The tester and driver cDNAs were digested with *Rsa*I, which is a four base-cutting restriction enzyme, to yield blunt ended cDNA fragments for adapter ligation.

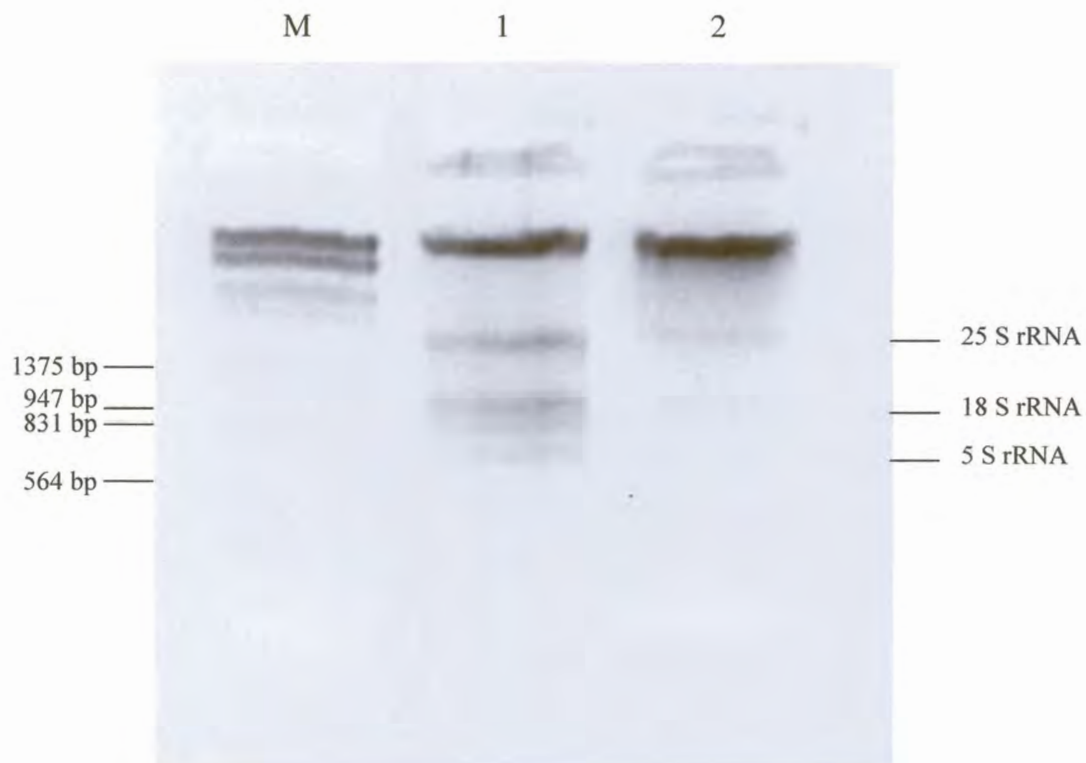

Figure 3.1 Total RNA isolated from the wheat accession PI 294994. M: Marker III is λDNA digested with *Eco*RI and *Hin*dIII. Lane 1: RNA from infested wheat. Lane 2: RNA from uninfested wheat.

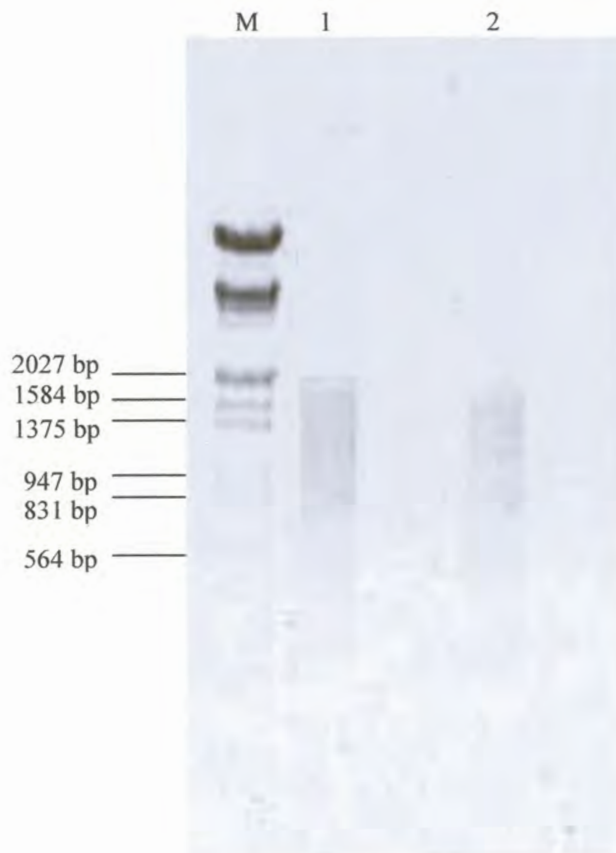

Figure 3.2 Double stranded cDNA synthesized from infested wheat line PI 294994 and cDNA obtained after *Rsa*I digestion. M: Marker III is λDNA digested with *Eco*RI and *Hin*dIII. Lane 1: cDNA after cDNA synthesis. Lane 2: cDNA after *Rsa*I digestion.

3.1.2 Adapter Ligation

The "tester" cDNA fragments were divided into two samples and ligated with two different cDNA adapters. The adapter ends were designed without phosphate groups so that only the longer strand of each adapter can be covalently attached to the 5'-ends of the cDNA. Therefore, there will be only one adapter per cDNA fragment. The two adapters have regions of identical sequence to allow annealing of the primary PCR primer, once the recessed ends have been filled in. The PCR performed after the adapter ligation, verified that some of the cDNAs had adapters on both ends. If the ligation is less than 25% complete, then the subtraction efficiency will be significantly reduced. The ligation test produced a smear of fragments extending from 0.5-2 kb (Fig. 3.3). The smear indicates the range of cDNA fragments that have adapters

ligated onto them and once the PCR was performed a region was amplified that spans the area that the adapters were attached to.

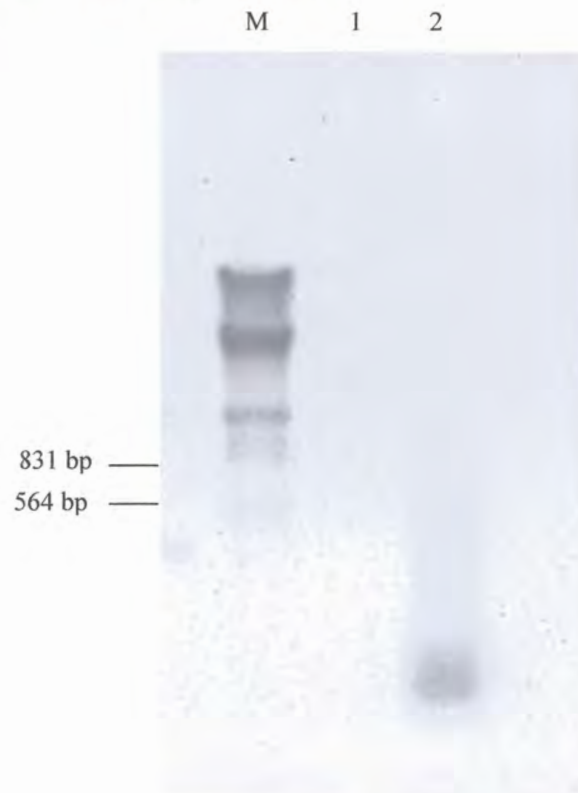

Figure 3.3 Ligation test showing range of cDNA fragments with ligated adapters. M: Marker III is λDNA digested with *Eco*RI and *Hin*dIII. Lane 1: Negative control. Lane 2: PCR product spanning adapter region.

3.1.3 Hybridization reactions and suppressive PCR

The SSH technique has two hybridizations. In the first hybridization an excess of "driver" was added to each sample of the "tester". During the hybridization the single stranded (ss) cDNA "tester" was normalized causing concentrations of low and high abundance cDNAs to become equal. After this equalization the tester fractions are combined in the presence of freshly denatured driver. During this second hybridization the differentially induced cDNAs are forced to hybridize with each other in compliance with second order hybridization kinetics (Diatchenko *et al.*, 1996). The resulting hybrids should include double stranded (ds) "tester" molecules with different adapter sequences on their 5'-ends, tester molecules with the same adaptor on either side, tester-driver hybrids, driver-driver hybrids, and single stranded molecules. In all PCR cycles, exponential amplification can only occur with the

differentially expressed "tester" sequences that have two different adapters. The PCR products amplified from the subtraction are shown in Fig. 3.4.

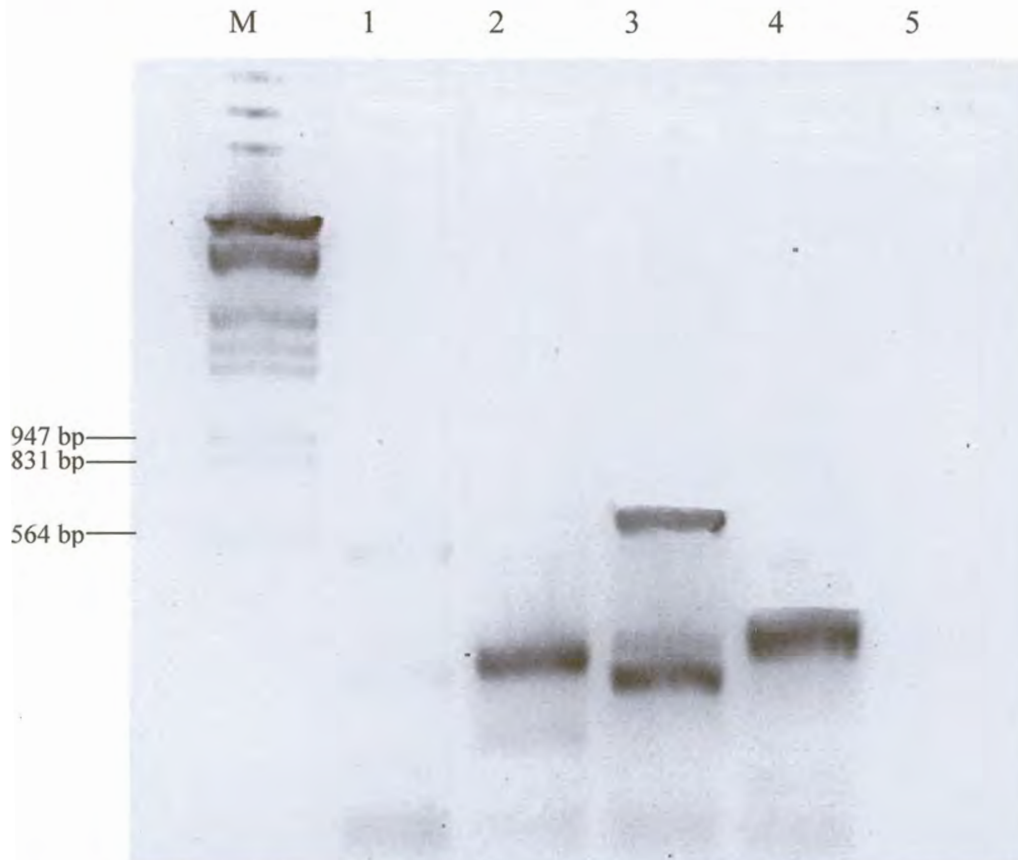

Figure 3.4 Secondary PCR products obtained after subtraction of PI 294994 cDNA. M: Marker III is λDNA digested with *EcoRI* and *HindIII.* Lane 1: Secondary PCR products using 2µl primary PCR as template. Lane 2: Secondary PCR products using 4µl primary PCR as template. Lane 3: Secondary PCR products using 5µl primary PCR as template. Lane 4: Secondary PCR products using 6µl primary PCR as template. Lane 5: Water control.

The primary PCR was carried out with a range of different volumes of template from the subtraction reactions. They were 2µl, 4µl, 5µl and 6µl respectively. There were no PCR products visible after the primary PCR. This is probably due to the very low concentrations used in the subtraction reactions. The 2µl sample generated three bands ranging in size from 130-300 bp, while the other samples generated two bands ranging from 170-350 bp in size. In total four different sized fragments were obtained from the secondary PCR amplification. These four fragments were cloned and

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characterized further by sequencing and sequencing analysis. The fragments were designated SSH1 to SSH4.

3.1.4 Sequencing Analysis

A total of four different clones were sequenced (Fig. 3.5). The sequences were translated to their amino acid equivalents and submitted to GenBank database searches, using the BLAST-N and BLAST-X algorithms (Altschul *et al*., 1997). The fragment designated SSH1, showed a strong similarity (E= 4e-06 and 3e-13) on DNA and protein sequence to an unknown protein from *Arabidopsis thaliana* (AY065284)(NP_190339). At the nucleotide level 38 out of the 41 bases were the same and at the amino acid level 38 out of the 73 amino acids were the same. At the protein level a similarity to the Burkitt lymphoma receptor was also identified (NP_445755) (Kouba *et al*., 1993). The SSH2 sequence indicated a similarity (E= 0.80) to a fibrinolytic enzyme mRNA from the earthworm, *Lumbricus rubellus* (AB045719) (Sugimoto and Nakajima, 2001), at the DNA level 23 out of 24 bases were the same. At the protein level the sequence indicated a similarity (E= 0.005) to a putative protein from *Arabidopsis thaliana* (NP_200056) with 22 out of 35 amino acids were the same. The SSH3 sequence showed a high similarity (E=1e-66) to *Triticum aestivum* mRNA for thioredoxin M. (AJ005840) (Capitani *et al*., 2000), with 133 out of 134 bases were the same. Another significant homology was to the heat shock protein gene from *Anopheles albimanus* (L47285) (Benedict *et al*., 1996) with an E-value of 0.44, with 20 out of 20 bases the same at the DNA level. However, at the protein level SSH3 contained one or more stop codons and thus could not be translated to a polypeptide. The SSH4 sequence was identical to *Triticum aestivum* 18s ribosomal RNA gene (AY049040). The other three sequences could be translated to polypeptides without any stop codons.

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4. Discussion

It has been found that the induction of a large number of defense-related genes is essential for plants to counter a wide range of pathogen infections. Many defense-related genes encode proteins possessing anti-fungal or antibacterial activities or enzymes that catalyze secondary metabolites linked to a defense response. Others encode regulatory proteins important for defense signal transductions (Xiao *et al.*, 2001). There are signal molecules present that induce a defense response and genes are expressed that activate a hypersensitive response-like programmed cell death (Jabs, 1999). The HR is associated with an oxidative burst, membrane damage, ion fluxes, endonuclease activation, DNA cleavage, plant cell wall modifications and pathogenesis-related (PR) protein expression, such as chitinase and glucanase (Lamb, 1994; Jackson and Taylor, 1996; Heath, 1998).

The first resistance (*R*) gene from plants was only cloned and characterized at the molecular level in 1992 (Staskawicz *et al.*, 1995). One of the most remarkable features discovered about the *R* genes in many different plant species is that it encodes proteins that have strikingly similar structures. The implication is that the defense response process is conserved between a wide variety of plant species (Bent, 1996). Sequence analysis has lead to the identification of a number of structural motifs found in resistance gene products. The structural domains found are serine-threonine kinases, leucine-rich repeats, nucleotide-binding sites, leucine zippers, Toll/interleukin-1 resistance domains and transmembrane receptor kinases (Rommens *et al.*, 1995; Bent, 1996; Jackson and Taylor, 1996; Keen, 1992). The isolation and characterization of these genes are essential for our understanding of plant disease resistance mechanisms.

An effective approach for studying the genetic nature of many biological processes is to characterize genes that vary in expression level during this process (Lisitsyn *et al.*, 1993; 1994). Suppression subtractive hybridization is a highly efficient and widely used PCR-based method for identifying differentially expressed genes. A key feature of the SSH technique is simultaneous subtraction and normalization that makes it possible to equalize the abundance of target cDNAs in the subtracted population.

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Thus, about a thousand fold enrichment is achieved for rare differentially expressed transcripts (Diatchenko *et al*., 1996). Success of SSH application experiments is limited by factors including high complexity cDNA samples and having a small number of differences between cDNA samples, such as is evident in wheat. So with the aid of SSH it was expected that some of the sequences obtained would show some similarity with either signal transduction molecules, PR proteins or structural motifs from resistance gene products.

In the wheat line PI 294994 the mode of action, the number and position of the Russian wheat aphid resistance genes has been identified (Liu *et al*., 2001). However, the genes themselves have not yet been fully identified and characterized. The complexity and genome size of wheat compared to other crops studied has limited the cloning of *R* genes (Leister *et al*., 1998; Seah *et al*., 1998). The sequences that were obtained in this study, mostly showed homology to genes that are linked to stress conditions in a range of organisms. The sequence designated SSH1, was very similar to others found in *Arabidopsis thaliana* that has as yet no known ascribed function. However, at amino acid level the sequence showed some similarity to a Burkitt lymphoma receptor (Kouba *et al*., 1993). The receptor plays a role in programmed cell death in human cancer cells and may indicate that SSH1 plays a role in a sort of programmed cell death in the wheat-aphid interaction. The SSH2 sequence at the nucleotide level is similar to a fibrinolytic enzyme, commonly found in snake venom, that causes the hydrolysis of fibrinogen (Sugimoto and Nakajima, 2001). There is a possibility that SSH2 is linked to localized cell death at the site of the aphid stylet insertion, during the hypersensitive response. The SSH3 nucleotide sequence shared homology to a *Triticum aestivum* mRNA for thioredoxin M. This regulates a wide range of enzymes by acting as a redox carrier, reversibly reducing and oxidizing disulphide bridges on the target protein (Capitani *et al*., 2000). Thioredoxins are part of the chloroplast regulatory system and thioredoxin M specifically regulates the activity of enzymes that are indirectly involved in photosynthesis, NADP-malate dehydrogenase, glucose-6-phosphate dehydrogenase and the CF1-ATPase. Another more significant similarity is that to the *Anopheles albimanus* heat shock protein gene (Benedict *et al*., 1996). The increased transcription of a set of genes in response to heat or other toxic agent exposure, is a highly conserved biological response that occurs in all organisms (Benedict *et al*., 1996). The response is mediated by heat

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shock transcription factor (HSF), which is present in a monomeric, non-DNA binding form in unstressed cells and is activated by stress to a trimeric form which can bind to promoters of heat shock genes. The heat shock element, a cis-element for activated heat shock factor, consists of inverted repeats of the sequence nGAAn, where n is an arbitrary nucleotide. This particular gene may be induced when the presence of the phytotoxin, injected by the aphid while feeding, is detected.

More sequences were expected to be present after the subtraction than was finally obtained. This could be explained in that a lot of the resistance response precursors or signalling pathway components are already present before the response is induced (Dangl *et al.*, 1996; Feuillet *et al.*, 1997). Therefore, by subtracting between induced and un-induced individuals, these transcripts would be removed and not detected. The two cDNA populations used in the subtraction may differ only in the relative amount of resistance transcripts and SSH will subtract out transcripts that are found in both populations, leaving only uniquely expressed sequences.

This study has opened a number of avenues, which may be pursued further to elucidate the response that was induced in the resistant wheat PI 294994. The unknown sequences that were identified would need to be characterised more and their possible role in the resistance response needs to be determined. Another improved approach would be to repeat the SSH experiments using an infested PI 294994 and an infested near isogenic wheat line without the resistance genes. A larger number of non-unique transcripts may be revealed but the previously mentioned problems encountered in this study could be avoided.

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CHAPTER IV

Isolation of Resistance Gene Analogs from PI 294994 Wheat Based on a Conserved Nucleotide-binding Site Motif

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1. Introduction

Disease resistance (*R*) genes have been isolated and characterized at the molecular level in several plant species such as *Arabidopsis*, tobacco, tomato and wheat (Jones and Jones, 1997; Cannon *et al.*, 2002). *R* gene products specifically recognize and provide gene-for-gene resistance towards a large number of pests and pathogens (Seah *et al.*, 1998; Pan *et al.*, 2000). *R* genes can be divided into four broad, structurally distinct classes. The first class of *R* genes belongs to the serine-threonine kinases (Martin *et al.*, 1993; Ritter and Dangl, 1996). The protein kinases phosphorylate the serine-threonine regions and thus control certain protein activities during the resistance response. The second class of *R* genes encodes putative transmembrane receptors with extracellular leucine rich repeat (LRR) domains (Jones *et al.*, 1994; Dixon *et al.*, 1998). The third class encodes for a receptor-like kinase and combines qualities of both the previous classes. Both the LRR domain and the protein kinase region are encoded in the same protein. The fourth class, which represents the majority of plant disease resistance genes cloned so far, is the nucleotide-binding site-leucine rich repeat (NBS-LRR) resistance genes. The NBS-LRR class of genes is abundant in plant species. In *Arabidopsis* it has been estimated that at least 200 different NBS-LRR genes exist making up to 1% of the genome (Ellis *et al.*, 2000; Sandhu and Gill, 2002).

The NBS-LRR genes contain three distinct domains: a variable N-terminus, a nucleotide-binding site and leucine rich repeats. Two types of N-termini are present in NBS-LRR. One kind of NBS-LRR contains a leucine zipper or coiled-coil sequence that is thought to facilitate the protein-protein interactions. The coiled-coil motif has been found in the N-terminus of both dicotyledons and cereals (Pan *et al.*, 2000; Cannon *et al.*, 2002). The second kind of N-terminus has been described only in dicotyledons and is similar to the cytoplasmic signaling domains on the *Drosophila* Toll- or the mammalian interleukin receptor-like (TIR) regions (Whitham *et al.*, 1994; Cannon *et al.*, 2002). The NBS regions are found in many ATP and GTP-binding proteins that act as molecular switches (Jackson and Taylor, 1996). These genes regulate the activity of proteases that can initiate apoptotic cell death. Since defense mechanisms in plants include the hypersensitive response, which is very similar to apoptosis, the common occurrence of NBS domains in both plants and animals could

be an indication of similar functioning.

NBS-LRR homologues encode proteins that are structurally closely related, this suggests that they have a common function in the signal transduction pathway, even though they confer resistance to a wide variety of pathogen types. The conservation between different NBS-LRR resistance genes enables the use of polymerase chain reaction (PCR)-based strategies in isolating and cloning other *R* gene family members or analogs using degenerate primers for these conserved regions. Strategies using degenerate primers have been successfully utilized in the cloning of other putative NBS-LRR resistance gene analogs (RGA) from potato (*Solanum tuberosum* L.)(Leister *et al*., 1996), soybean (*Glycine max* (L.) Merr.)(Yu *et al*., 1996) and citrus (Deng *et al*., 2000).

The aim of this chapter was to isolate and characterize NBS-LRR class resistance gene candidate sequences from the wheat line PI 294994, which contains the *Dn5* Russian wheat aphid resistance gene.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

All the chemicals used were of analytical grade.

2.1.2 Plant Material

Resistant wheat seed, PI294994 also named "Strelinskaja Mestnaja", is a hard winter wheat that originated in Hungary but was acquired from CIMMYT (Mexico). The plants were grown in pots under greenhouse conditions and prevailing day and night cycles at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. The temperature was maintained at about 24°C. Plants were watered daily. Half of the wheat seedlings were infested with RWA at the 3-4-leaf growth stage with about 10 aphids per plant. After a week, leaves from the uninfested plants and all heavily infested leaves were removed. The aphids were removed from the infested leaves under running water to prevent aphid derived nucleic acid contamination during the RNA isolation. The leaves were dried and used immediately for total RNA isolation.

2.1.3 Treatment of glassware, plastic ware and solutions

All glassware, plastic ware and solutions used, up to the second strand cDNA synthesis, were treated and then kept free of RNases. All glassware was treated overnight in 0.1% (v/v) diethyl pyrocarbonate (DEPC), autoclaved for 20 min at 121°C and baked at 200°C for 3-4 hours (Sambrook *et al.*, 1989). The mortars and pestles were washed in 0.25M HCL for 30 min, prior to DEPC treatment, autoclaving and baking. All plastic ware and solutions, except those containing Tris (2-Amino-2-(hydroxymethyl)-1,3-propandiol), were DEPC treated and autoclaved.

2.2. Methods

2.2.1 Total RNA Isolation

Total cellular RNA was extracted by a modified method of acid guanidium thiocyanate-phenol-chloroform extraction described by Chomczynski and Sacchi (1987). All procedures were executed at 0-4°C. The infested and uninfested wheat

leaves were separately ground to a powder in liquid nitrogen with a mortar and pestle. Extraction buffer (4M guanidine thiocyanate, 25mM sodium citrate, 0.5% (w/v) N-lauroylsarcosine and 100mM 2-mercaptoethanol added just before use) was added to the homogenate in a 3:1 volume to tissue ratio. Successively, 2M sodium acetate (pH 8.00), Tris-EDTA (pH 8.00) equilibrated phenol and chloroform: acetic acid (49:1) was added and vortexed after each addition. The samples were incubated for 15 min on ice and centrifuged at 10 000xg for 20 min. An equal volume of isopropanol was added to the supernatant and the nucleic acids precipitated for 1.5-5 hours at -20°C. The samples were centrifuged at 10 000xg for 20 min and the pellet was resuspended in extraction buffer and precipitated with an equal volume of isopropanol at -20°C for 1 hour. The centrifugation was repeated as described and the pellet washed 3 times with ice-cold 75% (v/v) ethanol. The pellet was resuspended in ethanol, centrifuged and dried under vacuum and finally dissolved in RNase free water. The RNA samples were stored at -80°C for further use.

2.2.2 RNA Concentration Determination

The RNA concentration was determined on a Beckman DU®-64 spectrophotometer, by reading the absorbance at 260 nm and calculated using:

$$OD_{260} \times 40\mu g.ml^{-1} \times \text{dilution factor} = \text{concentration RNA } (\mu g.ml^{-1})$$

The 260/280 ratio was determined to indicate the level of protein contamination (Sambrook *et al.*, 1989).

2.2.3 Agarose gel electrophoresis analysis of RNA isolation

The integrity of the RNA was tested by analyzing both the infested and uninfested total RNA on a 2% (w/v) agarose gel (Sambrook *et al.*, 1989). The system was run with a 1x TAE electrophoresis buffer (0.08M Tris-acetate, 0.002M EDTA pH 8.00) and the samples mixed with 6x loading buffer (15% Ficoll, 0.25% bromophenol blue) prior to loading of the gel. The molecular mass standard that was used is λ DNA digested with *Eco*RI and *Hin*dIII (Sambrook *et al.*, 1989). The RNA isolation products were developed at 100 V for 30 min and visualized under UV light with ethidium bromide (EtBr) staining.

2.2.4 mRNA Isolation

The mRNA was purified from the total RNA using Oligo(dT) Cellulose affinity chromatography (GibcoBRL, Life Technologies). A column of 1g of oligo(dT) cellulose was poured in a Pasteur pipette plugged with sterile glass wool. The total RNA was dissolved in TE buffer (10mM Tris-HCl pH 7.40, 1mM EDTA), heat denatured at 65°C for 5 min, applied to the column and run through under gravity. The columns were then centrifuged at 350xg for two min, a high salt buffer (10mM Tris-HCL pH 7.40, 1mM EDTA, 0.5M NaCl) was added and centrifuged again for 2 min. The column was washed three times with a low salt buffer (10mM Tris-HCl pH 7.40, 1mM EDTA, 0.1M NaCl) and the mRNA was eluted with four volumes of elution buffer (10mM Tris-HCl pH 7.40, 1mM EDTA) that was pre-warmed to 65°C. The eluate was collected by centrifugation for two min. The synthesis of cDNA was carried out using the Roche Molecular Biochemicals cDNA Synthesis System according to manufacturer's specifications. Both the uninfested and the infested wheat mRNA was used as the substrate for the cDNA synthesis reaction. The ds cDNA was purified by the QIAquick Spin Purification Procedure (Qiagen). The cDNA was eluted in water and the concentration determined spectrophotometrically and stored at -20°C.

2.2.5 RNA ligase-mediated rapid amplification of 5' and 3' cDNA ends (RLM-RACE)

The mRNA was dephosphorylated with calf intestinal phosphatase (CIP) to remove the 5' phosphates and decapped with tobacco acid pyrophosphatase (TAP) to remove the 5' cap. The dephosphorylated, decapped mRNA was ligated to a GeneRacer™ RNA oligo using the GeneRacer Kit (Invitrogen). The ligated mRNA was reverse-transcribed using SUPERSCRIPT™ II RT (Invitrogen) and the GeneRacer™ Oligo dT primer to create RACE-ready cDNA with known priming sites at the 5' and 3' ends. The 5' ends were amplified using a reverse degenerate nucleotide-binding site primer and the GeneRacer™ 5' Primer. The degenerate oligonucleotide primers were based on the amino acid sequences of two highly conserved motifs of the NBS in the tobacco *N* and *Arabidopsis RPS2* genes (Yu *et al.*, 1996). The 3' ends were amplified using a forward degenerate nucleotide-binding site primer and the GeneRacer™ 3' primer (GCTGTCAACGATACGCTACGTAACGGCATGACAGTG$(T)_{18}$). The

cycling parameters used for the GeneRacerTM reactions were five cycles consisting of 94°C for 30 sec and 72°C for 1 min, five cycles of 94°C for 30 sec, 70°C for 30 sec and 72°C for 1 min and twenty cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 1 min.

2.2.6 Degenerate NBS-PCR

For the amplification of NBS sequences using the degenerate primers NBS-F1 (GGAATGGGNGGNGTNGGNAARAC) and NBS-R1 (YCTAGTTGTRA-YDATDAYYYTRC), where R = A/G, Y = C/T, D = A/G/T, H = A/C/T, N = A/G/C/T, were used. The PCR reaction consisted of 50μM of each primer, 50ng of the RT template, 1X reaction buffer (Promega), 2.5mM $MgCl_2$, 0.2mM of each dNTP and 2.5U of *Taq* DNA polymerase, and 1.3M betaine to increase primer annealing. Thirty cycles of PCR, consisting of 95°C for 1 min, 55°C for 1.5 min, and 72°C for 1 min, were performed in a Perkin-Elmer GeneAmp PCR System 9700 DNA thermal cycler (Applied Biosystems).

2.2.7 Cloning and Analysis of NBS-PCR Products

The PCR products were purified from an agarose gel slice using a Geneclean II Kit (Bio101). These fragments were cloned into the pGEM®-T Easy vector system (Promega). Ligation mixtures were used to transform competent *E. coli* (JM109) cells. Plasmid DNA was isolated from candidate clones and purified. Sense and antisense strands of the clones were used in cycle sequencing using the dideoxy-DNA chain-termination method with the BigDye Terminator Cycle Sequencing Reaction kit (Perkin-Elmer) on the ABI-3100 Prism Automated sequencer (Perkin Elmer). The sequences were used in BLAST searches and they were aligned to other published sequences in GenBank (Altschul *et al*., 1997).

2.2.8 Southern hybridization of segregating wheat populations

The wheat lines and the F_2 populations used in this Southern hybridization were developed at the Small Grain Institute, Bethlehem, South Africa. The near-isogenic lines (NILs) namely Palmiet *Dn1*, Palmiet *Dn2* and Palmiet *Dn5*, were developed by backcrossing the *Dn1*, *Dn2* or *Dn5* source lines (SA 1684, SA 2199 and SA 463, respectively) to the susceptible Palmiet spring cultivar six times. The resulting

segregating lines were SA 1684/6*Palmiet, SA 2199/6*Palmiet and SA 463/6*Palmiet respectively. Crossing the original resistant parent with the susceptible Palmiet developed a separate F_2 population. The segregation analysis of the F_2 population was determined previously (Myburg *et al.*, 1998). Genomic DNA samples of the segregating populations (10μg) were digested separately overnight with restriction enzymes, *Eco*RI, *Hin*dIII, *Eco*RV, *Bam*HI and *Pst*I. The digested gDNA was then electrophoresed on a 1% (w/v) agarose gel at 60 V for 60 min. The resulting smears were transferred to a positively charged nylon membrane (Micro Separations Inc.) by the Southern blot technique (Sambrook *et al.*, 1989). The DNA was cross-linked to the nylon membrane using a UV cross-linker (UVtec) at 1.5 J/cm^2 for 5 min. A 519 bp NBS fragment obtained from the degenerate PCR was DIG-labeled and used to probe the membrane with a hybridization temperature of 45°C (Roche Diagnostics).

2.2.9 RNA dot blot

Total RNA was isolated from the root, leaf, stem and seed head of the wheat line PI 294994 of an uninfested individual. The RNA dot blot was carried out with a vacuum manifold (Bio-Rad) that had been cleaned with 0.1N NaOH. Samples of about 1μg total RNA were loaded and allowed to absorb to a positively charged membrane (Micro Separations Inc.) under a gentle vacuum. The RNA was cross-linked as before. The DIG-labeled 519 bp NBS fragment obtained from the degenerate PCR was used to probe the RNA dot blot with a hybridization temperature of 45°C (Roche Diagnostics).

2.2.10 DNA extraction

DNA was extracted according to a modified extraction method of Edwards *et al.* (1991). Leaf samples of approximately 10g were ground to a fine powder in liquid nitrogen with a mortar and pestle. After all of the nitrogen had evaporated two volumes of warm (65°C) extraction buffer (100mM Tris-HCl pH 8.00, 500mM NaCl, 50mM EDTA, 1.25% (m/v) SDS, 20mM $Na_2S_2O_5$,) was added to the still frozen tissue and vortexed. After 30 min at 65°C, one volume of chloroform/isoamylalcohol (24:1) was added and the mixture was vortexed vigorously until white foam formed. The emulsion was centrifuged for 15 min at 12 000xg and the upper phase transferred

to a clean 15ml tube. This tube was filled to the top with ice-cold 100% ethanol and after 12 hours the DNA was scooped out, washed in 70% ethanol and air-dried. The dry DNA was dissolved in 200µl sterile water and the concentration determined spectrophotometrically. The samples were then aliquoted and stored at -20°C.

2.2.11 Southern hybridization and fragment detection

Genomic DNA samples of wheat line PI 294994 (10µg) were digested separately overnight with restriction enzymes, *Eco*RI, *Hin*dIII, *Eco*RV, *Bam*HI and *Pst*I and transferred to a nylon membrane as previously described. A 464 bp fragment obtained using the degenerate NBS-PCR was DIG-labeled and used to probe the membrane with a hybridization temperature of 45°C (Roche Diagnostics).

2.2.12 Inverse PCR and sequence analysis

To amplify the remainder of the NBS gene, a modified inverse PCR technique of Ochman *et al.* (1990) was used. Regions of genomic DNA corresponding to the positive hybridization signals in the *Hin*dIII, *Eco*RV and *Bam*HI digests were excised from the gel and purified using the QIAquick gel extraction kit (Qiagen). The purified smears were self-ligated with T4 DNA ligase overnight (Roche Diagnostics). Specific inverse primers were designed from the 464 bp NBS-DNA fragment. The inverse primers were designed to amplify the remainder of the NBS gene. In the inverse PCR reactions 0.6µM per reaction of primer IPSL3 (AGAAGGGATGAGGTGGGTAAG) and IPSL4 (AGAAAGGATGGGG-AGGGTAAG) were used to amplify 5ng of the circularized PI 294994 template DNA. The PCR reaction consisted of 1X reaction buffer (Promega), 1.5mM $MgCl_2$, 0.2mM dNTPs and 5U of *Taq* DNA polymerase. Thirty cycles of PCR, consisting of 94°C for 30 sec, 50°C for 30 sec and 72°C for 2 min. The reactions had an initial denaturation step of 4 min at 94°C and a final elongation of 5 min at 72°C. The PCR was performed in a Perkin-Elmer GeneAmp PCR System 9700 DNA thermal cycler (Applied Biosystems). The fragment products from the inverse PCR were purified from the agarose gel, cloned and analyzed as before.

2.2.13 Walking in uncloned genomic DNA

The second technique used to amplify the remainder of the NBS gene was a modified version of the PCR method for walking in uncloned genomic DNA used by Siebert *et al.* (1995). Regions of genomic DNA corresponding to the positive hybridization signals in the *Hin*dIII, *Eco*RV and *Bam*HI digests were excised from the gel and purified using the QIAquick gel extraction kit (QIAGEN). The ends were filled in with dNTPs at 70°C for 15 min and the DNA was ligated to an excess of walking adapter (CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT) over-night at 16°C under the following conditions: 50mM Tris-HCl pH 7.60, 10mM MgCl, 0.5mM ATP, 10mM dithiothreitol, 5µl adapter and 10U T4 DNA ligase (Roche Diagnostics) in a total volume of 10µl. The ligation reaction was terminated by incubation of the tubes at 70°C for 5 min after which the reaction was diluted ten-fold and stored at -20°C. PCR amplifications were performed using the Expand High Fidelity PCR System (Roche Diagnostics). Primary PCR reactions were conducted in 50µl volumes containing 1µl template DNA, 0.2mM of each dNTP, 0.4µM adapter primer AP1 (AGAAACCCGACCTACCACGGCTTGCTCCTT), inverse NBS primer (IPSL3 or IPSL4) and 1x Expand HF buffer with 1.5mM $MgCl_2$ and 2.6U enzyme mix. The PCR reaction was performed by initial denaturation of 1 min at 94 °C, followed by 25 cycles of 94°C for 30s, 55°C for 30s, 68°C for 4 min, and a final elongation of 15 min. A secondary PCR reaction was conducted with 1µl of a 100-fold dilution of the primary PCR using adapter primer AP2 (AATAGGGCTCGAGCGGC) and the inverse NBS primer (IPSL3 or IPSL4). The same reaction composition and cycle parameters were used. PCR products were examined on a 1% agarose/EtBr gel.

3. Results

3.1 RNA ligase-mediated rapid amplification of 5' and 3' cDNA ends

3.1.1 RACE of the NBS gene analog sequence

The amplification of the cDNA ends of the NBS gene analog sequence produced smears of fragments extending from about 2 kb to less than 100 bp (Fig. 4.1). There were indistinct bands visible in the smears and a secondary PCR was attempted on the samples but no clearer results were obtained. The results indicated the gene specific primer (GSP) namely the degenerate NBS primers, needed to be redesigned with a 50-70% GC content to obtain a high annealing temperature of over 70˚C. Also no sequence of the gene of interest was known thus a degenerate PCR approach was adopted.

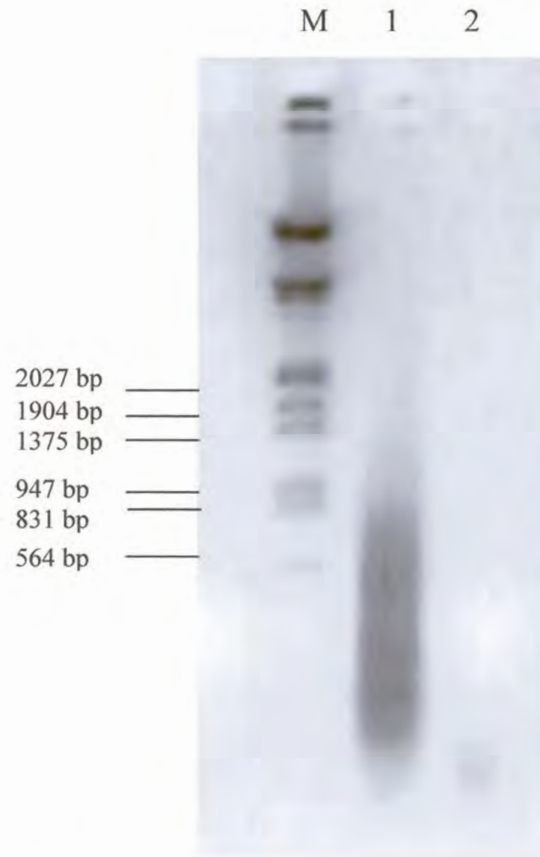

Figure 4.1 Amplification of RACE cDNA ends. M: Marker III is λDNA digested with *Eco*RI and *Hin*dIII. Lane 1: NBS gene cDNA ends. Lane 2: Negative control.

3.1.2 Degenerate NBS-PCR

NBS analog sequences containing genomic sequences were produced by the amplification of the GeneRacer™ cDNA using degenerate PCR primers described in section 2.2.6. Fragments in the 500 bp size range were obtained from the wheat PI 294994 (Fig. 4.2). After cloning, three different sized fragments were obtained. These three fragments were characterized further through sequence analysis.

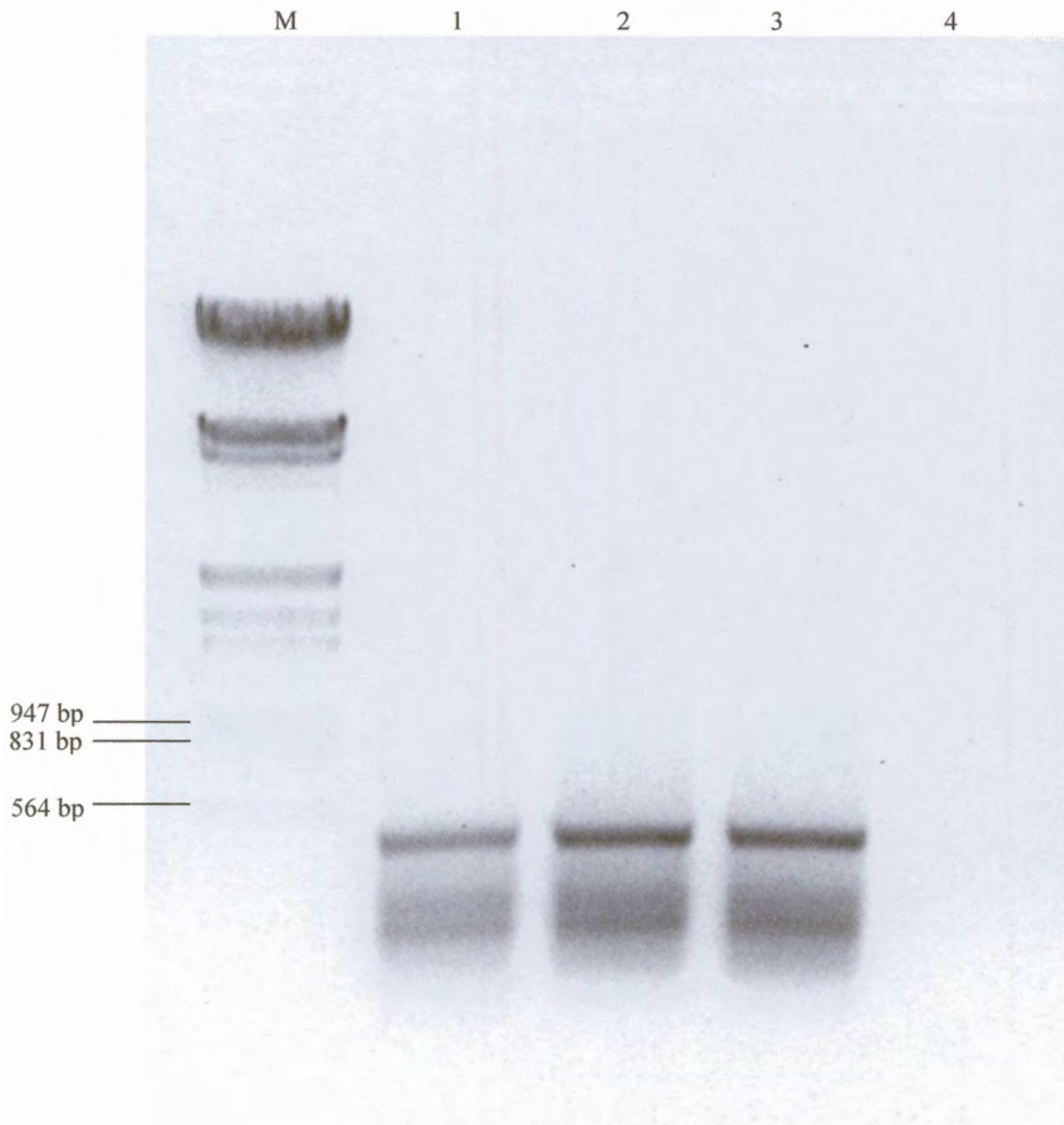

Figure 4.2 Degenerate NBS PCR. M: Marker III is λDNA digested with *Eco*RI and *Hin*dIII. Lane 1: Primary PCR products. Lane 2: Secondary PCR products. Lane 3: Secondary PCR products with added Betaine. Lane 4: Negative control.

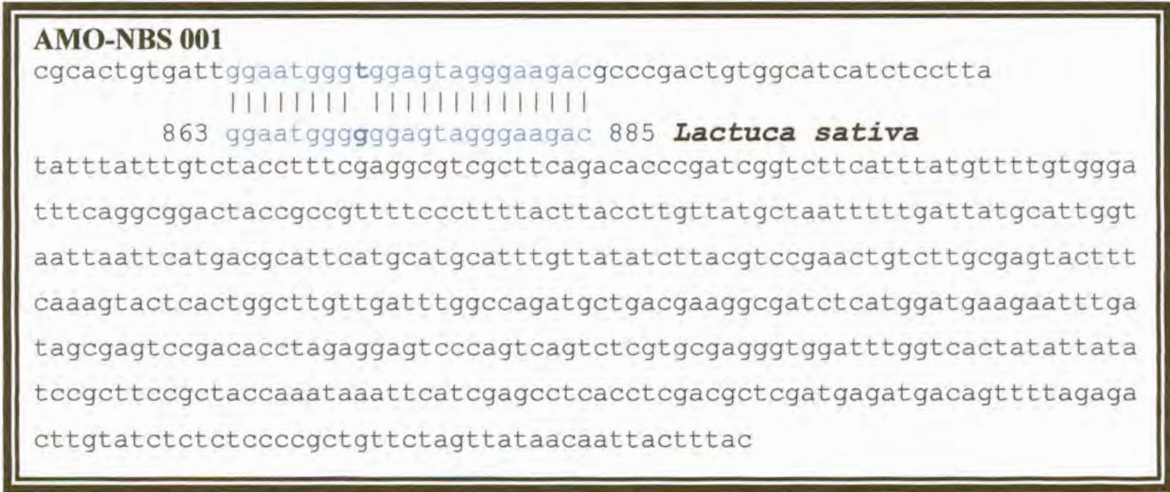

Figure 4.3 Nucleotide sequence alignments of degenerate NBS PCR.

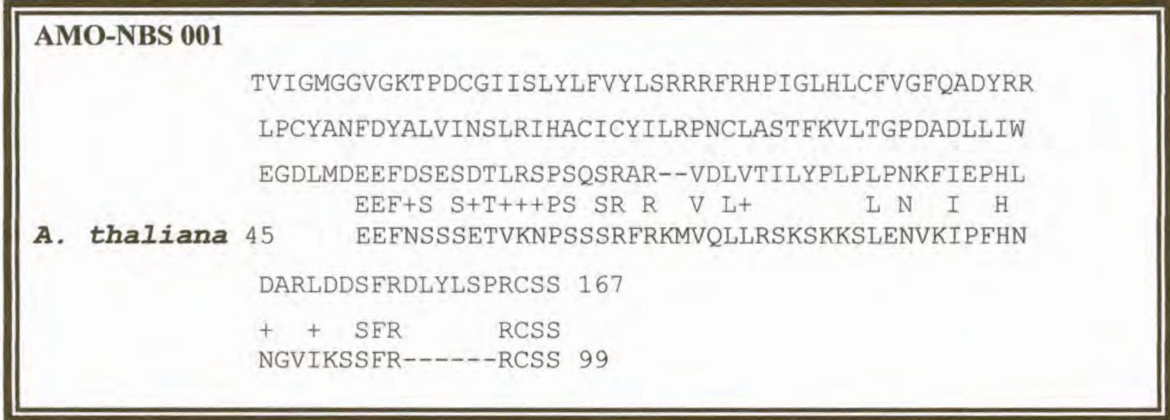

Figure 4.4 Amino acid sequence alignments of degenerate NBS-PCR.

3.1.4 Southern blot of segregating populations

Hybridization analysis of the segregating populations of the *Dn1*, *Dn2* and *Dn5* resistance genes with the NBS fragment displayed linkage to *Dn2* (Fig. 4.5). This fragment is present only in the resistant samples. In the other, *Dn1* and *Dn5*, segregating populations the fragment is found in almost all of the samples, both susceptible and resistant. In all cases the positive hybridization signal was present at about 3.2 kb in size.

Figure 4.5 Segregating F_2 wheat populations and parental lines. M: Marker III is λDNA digested with *Eco*RI and *Hin*dIII. Lanes 1-5: Susceptible F_2 individuals. Lanes 6-20: Resistant F_2 individuals. P: Palmiet. P_1: SA 1684/6*Palmiet (Palmiet *Dn1*). P_2: SA 2199/6*Palmiet (Palmiet *Dn2*). P_5: SA 463/6*Palmiet (Palmiet *Dn5*). SA_1: SA 1684. SA_2: SA 2199. SA_5: SA 463. Arrow indicates positive hybridization signals at 3.2 kb.

3.1.5 RNA dot blot

To determine in which tissues the isolated NBS gene was expressed, a RNA dot blot was performed using various tissues of the wheat PI 294994. When total RNA from the root, leaf, stem and seed head of the wheat line PI 294994 was probed with the DIG-labeled NBS fragment, positive signals were obtained in the stem, leaf and seed RNA samples (Fig. 4.6).

Figure 4.6 RNA dot blot of various wheat tissue types. Dot 1: Seed RNA. Dot 2: Root RNA. Dot 3: Stem RNA. Dot 4: Leaf RNA.

Another 464 bp NBS related fragment was obtained from the clones blue/white selection plate that showed homology (E = 0.006) to *Oryza sativa* NBS-LRR type resistance protein (*r15*) gene (AF032702) (Leister *et al*., 1998) (Fig. 4.7) and to *Lactuca sativa* resistance protein candidate RGC2A pseudogene (E = 1.4) (AF072268) (Meyers *et al*., 1998).

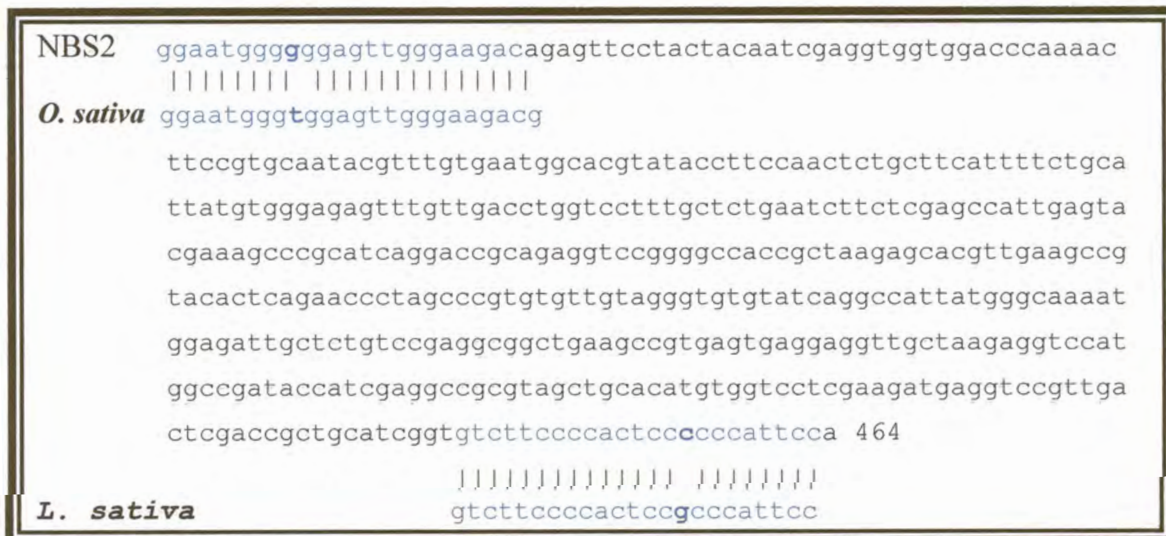

Figure 4.7 Nucleotide sequence alignments of degenerate NBS-PCR.

3.1.4 Southern hybridization and inverse PCR

The 464 bp NBS fragment obtained from the degenerate PCR produced positive signals in only three of the five restriction enzyme digested gDNA samples (Fig. 4.8). The *Hin*dIII and *Bam*HI digested gDNA produced signals at about 10 kb. The smaller fragment size, produced when gDNA was digested with *Eco*RV, at about 3.2 kb was selected for further analysis to facilitate circular ligation for inverse PCR, the larger fragments would hinder the process. The inverse PCR produced a major band at about 1.5 kb in size and other minor bands from 1 kb and smaller (Fig. 4.9). The larger major band was selected for subsequent cloning and sequencing. Thus, the remaining 5' and 3' portions of the PI 294994 NBS-LRR were determined by sequencing the inverse PCR products and this sequence contained overlapping regions with the previously sequenced 464 bp fragment.

Figure 4.8 Hybridization of DIG labeled NBS fragment to restriction enzyme digested gDNA. M: Marker III is λDNA digested with *Eco*RI and *Hin*dIII. Lane 1: *Eco*RI digested gDNA. Lane 2: *Hin*dIII digested gDNA. Lane 3: *Eco*RV digested gDNA. Lane 4: *Bam*HI digested gDNA. Lane 5: *Pst*I digested gDNA.

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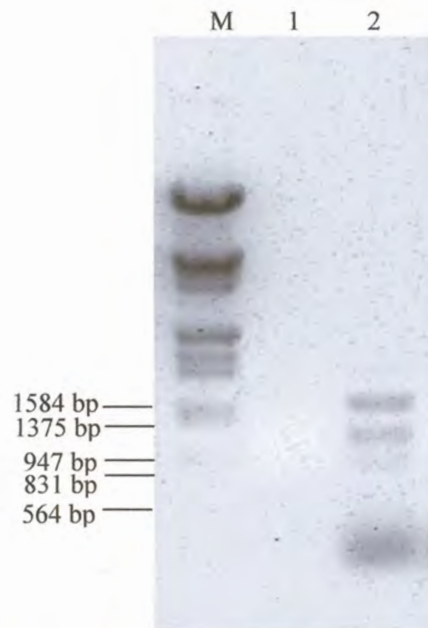

Figure 4.9 Inverse PCR. M: Marker III is λDNA digested with *Eco*RI and *Hin*dIII. Lane 1: Negative control. Lane 2: Secondary inverse PCR products.

3.1.5 Walking in uncloned genomic DNA

The DNA walking method produced a smear of fragments extending from about 3.2 kb downwards (Fig. 4.10). A number of major bands were visible in the smear. However, after numerous PCR optimization steps and secondary PCRs it was still not possible to produce single major PCR products.

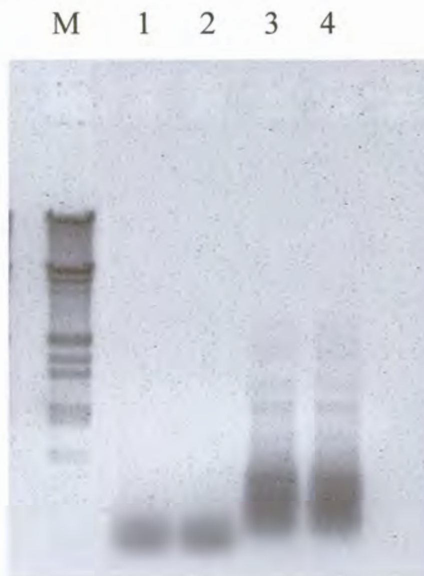

Figure 4.10 Amplification products generated from walking in uncloned genomic DNA. M: Marker III is λDNA digested with *Eco*RI and *Hin*dIII. Lane 1 and 2: Negative control. Lane 3 and 4: NBS gene cDNA ends.

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3.1.6 Sequence analysis

A total of three different clones were sequenced (AMO 004; AMO 005; AMO 006). After the primer sequences were removed, fragments of about 300 bp were analyzed. All of the fragments gave homology (E=0.0, and E=e-130) to a portion of the *E. coli* K12 MG1655 genome) (AE000136) (AE000168) (AE000464) (Blattner *et al.*, 1997 (Fig. 4.11). The sequences were translated in all three reading frames and aligned to protein sequences in the BLAST database (Altschul *et al.*, 1997). The first two fragments, AMO 004 and AMO 005, showed homology (E=0.12 and 6e-31) on the protein level to conserved hypothetical proteins (NC_003047)(NC_002655), and the third fragment, AMO 006, showed homology (E = 0.086) to the polymyxin β resistance protein PBS2 (S56909) in the yeast, *Saccharomyces cerevisiae* (Fig. 4.12)

```
AMO 004:     16 ggtaagggcctcattgaacggggcgttggccagaagccatcagctgctg
                |||||||||||||||||||||||||||||||||| |||||||||||||||
E. coli:13345   ggtaagggcctcattgaacggggcgttggccaga-gccatcagctgctg

                tcgagtttgttcgctgacactggcgttatcttcactctcttccagctgt
                |||||||||||||||||||||||||||||||||||||||||||||||||
                tcgagtttgttcgctgacactggcgttatcttcactctcttccagctgt

                atttccggatgcgtagttgtccatcgtccagccagataaaagcgctgc
                |||||||||||||||| |||||||||||||||||||||||||||||||
                atttccggatgcgtagctgtccatcgtccagccagataaaagcgctgc

                cgattcgctgatcgtcctggcgaccctggctaccggcgagatgaatata
                |||||||||||||||||||||||||||||||||||||||||||||||||
                cgattcgctgatcgtcctggcgaccctggctaccggcgagatgaatata

                gagcgggacgctcatgccgtcctgaagcgcctgactgaaggcctgcgga
                |||||||||||||||||||||||||||||||||||||||||||||||||
                gagcgggacgctcatgccgtcctgaagcgcctgactgaaggcctgcgga

                ataatcaccccacctatttgctgcgcacttatgtcagcagcgctggcat
                |||||||||||||||||||||||||||||||||||||||||||||||||
                ataatcaccccacctatttgctgcgcacttatgtcagcagcgctggcat

                cgggctgaacgaacaaaaagaccatgccgaaggcaaactgggctttcag
                |||||||||||||||||||||||||||||||||||||||||||||||||
                cgggctgaacgaacaaaaagaccatgccgaaggcaaactgggctttcag

                tcctggggaga 369
                |||||||||||
                tcctggggaga 13697
```

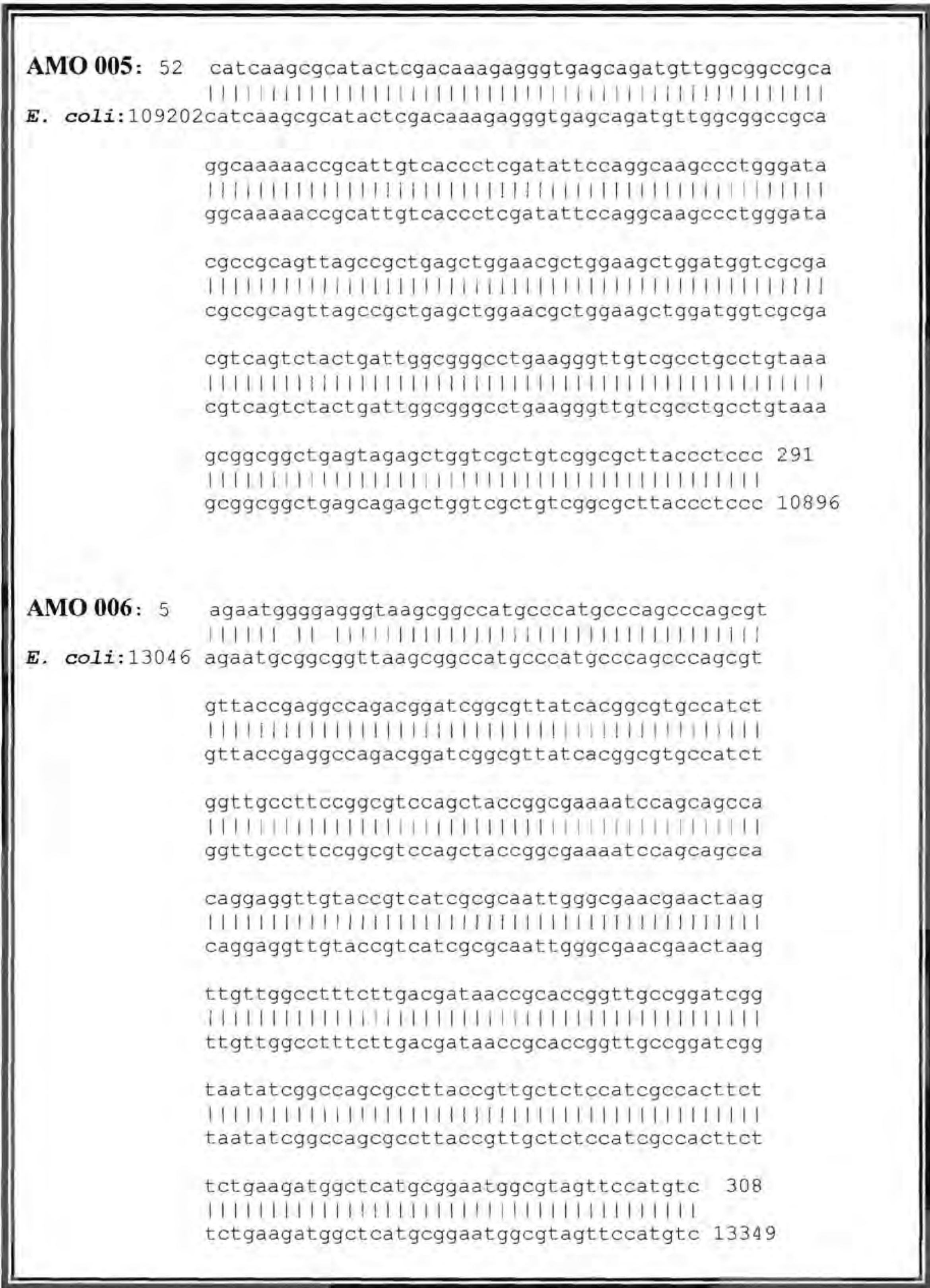

Figure 4.11 Nucleotide sequence alignments of inverse PCR.

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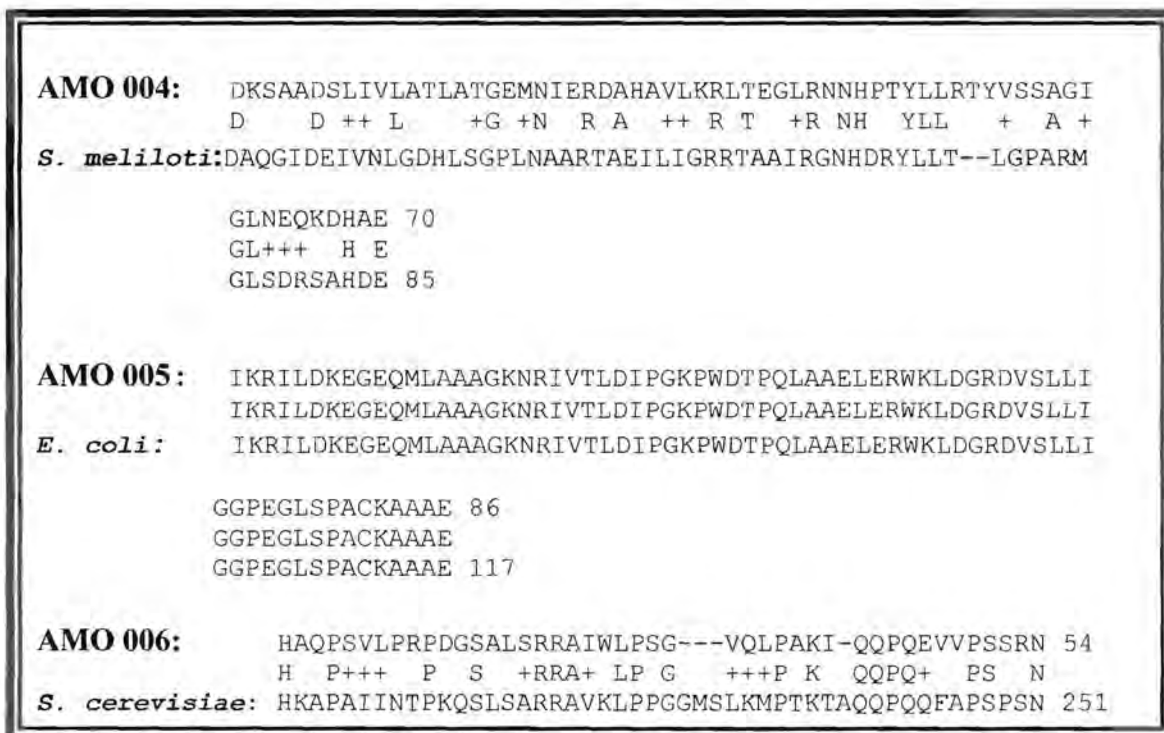

Figure 4.12 Amino acid sequence alignments of inverse PCR.

4. Discussion

The majority of plant disease resistance (*R*) genes cloned so far contain nucleotide-binding sites (NBS) and a leucine-rich repeat (LRR) domain. This class of *R* genes belongs to a superfamily that is present in both dicotyledons and monocotyledons as suggested from sequence comparisons made between these isolated genes (Bent *et al.*, 1994; Lagudah *et al.*, 1997). The use of PCR based approaches with degenerate oligonucleotide primers designed from the NBS region of cloned disease resistance genes has led to the cloning of resistance gene-like sequences in several plant species (Leister *et al.*, 1998; Seah *et al.*, 1998; Garcia-Mas *et al.*, 2001). Correlation or co-segregation of some of these sequences with known disease resistance gene loci has been reported.

The conserved NBS and LRR domains among several disease-resistance genes has led to the hypothesis of cloning additional resistance genes based on the homology to these conserved sequences. This hypothesis can be complicated by an excess of genes that contain the NBS region but are not related to resistance genes (Yu *et al.*, 1996). Also many homologous resistance genes may be located throughout the genome in a plant species. Thus, the sequence homology among these genetically independent and functionally distinct disease-resistance genes will present a difficulty in isolating individual clones, which correspond to a specific resistance gene by hybridization. However, it is useful when in the process of isolating as many NBS-LRR regions as possible for further study. Lastly, if multiple NBS-LRR-resistance genes exist as clustered multigene families on a chromosomal region, a practical method will be needed to distinguish different members of the multigene family and to match them to resistance genes or clusters, such as a genomic DNA library

A PCR approach, based on degenerate oligonucleotide primers designed from conserved motifs in the NBS domain, was used to clone disease resistance gene homologues from wheat line PI 294994. The RACE technique was attempted using one GeneRacer^{TM} primer and one degenerate NBS primer to isolate full-length expressed resistance genes. However, even after multiple attempts no clear bands were observed. The reason there was a smear and not a distinct band may be due to

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the extreme annealing temperatures (70°C) of the GeneRacer™ PCR causing nonspecific binding of the degenerate NBS primer. Thus a straightforward degenerate PCR approach was adopted. The cDNA that was generated from the GeneRacer™ technique was subjected to PCR with both degenerate NBS primers. Of the three different sized fragments obtained only the one showed a homology to NBS-LRR. The sequence provided a significant homology to a lettuce (*Lactuca sativa*) resistance protein candidate gene (Meyers *et al*., 1998).

The RNA dot blot using the obtained sequence as a probe revealed that the gene is strongly expressed in the wheat leaf and seed, to a lesser extent in the stem and not at all in the roots of the PI 294994 wheat plant. The results are rather unexpected since it had been assumed that the NBS-LRR expression is found in all tissue types and is present all of the time. Further studies using northern blots or real-time PCR will need to be carried out to determine if this is true or just an anomaly.

A southern blot of segregating wheat populations was carried out to identify any linkage that may exist between the NBS-containing gene and the Russian wheat aphid resistance genes *Dn1*, *Dn2* and *Dn5*. The southern blot showed that there is a very weak linkage with the *Dn2* resistance gene because the NBS probe hybridized with some of the *Dn2* resistant near isogenic lines (NILs) and not with any of the susceptible NILs. However, the NBS probe did not hybridize in any of the *Dn2* parental lines, Palmiet and SA 2199. In the *Dn1* and *Dn5* NILs the gene was found in almost all of the lines, both resistant and susceptible, so the NBS fragment was not linked to either of these RWA resistance genes.

The following aim was to determine the full-length cDNA sequence of the NBS-containing gene. Unfortunately the original clone was lost and another NBS clone was chosen. The sequence of the new NBS clone was slightly different to that of the previous one but it also showed homology to *Lactuca sativa* NBS-LRR. To determine the full-length cDNA sequence of this NBS-containing gene, an inverse PCR technique was employed as well as genome walking. After numerous rounds of PCR optimization and secondary PCRs no single major PCR products were generated in the genome walking. It is essential to produce single major PCR products because multiple PCR products complicate further characterization without southern blot

hybridization or extensive cloning. The inverse PCR produced a fragment 1.5 kb in size. When the fragment was sequenced there was no homology to any disease resistance genes at the nucleotide level, only to a portion of the *E. coli* genome. However, at the amino acid level there was some indication of homology to a kinase type resistance protein from *Saccharomyces cerevisiae*. A possible reason for the lack of nucleotide level homology being that the original 519 bp degenerate PCR fragment showed sequence and amino acid homology to resistance genes in areas other than the primer region, while the new 464 bp degenerate PCR fragment showed sequence homology at the beginning and end of the sequence. Thus, it is possible that the second clone that was obtained was not actually from an NBS-resistance gene at all but an NBS region that is not related to a disease resistance. Another compounding problem is that with the NBS region nucleotide sequences between species is quite different and even at the amino acid level an NBS clone is considered to be highly homologous to other NBS containing genes with similarities of between 14-32% (Yu *et al*., 1996).

Although the cloning of NBS-containing sequences was not very successful for cloning disease resistance gene homologues in this case, it is a technique that holds great promise for accelerating the process of identification of markers for known disease resistance genes. However, the use of degenerate oligonucleotide primers may be more sensitive in isolating conserved sequences but could also result in the identification of numerous genes that have a common conserved domain or nearly similar nucleotide sequence that has nothing to do with disease resistance.

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CHAPTER V

Summary/ Opsomming

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Summary

Infestation by the Russian wheat aphid, *Diuraphis noxia* (Mordvilko), has caused large-scale damage to small-grain crops since its introduction into South Africa in 1978 and many other countries world-wide. The extreme damage caused by *D. noxia* in wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) has resulted in a concerted effort by scientists to investigate the mechanisms and genes involved in the resistance response. This is done in an effort to understand and ultimately use the knowledge of resistance responses to produce aphid resistant crops. The aim of the present study was to use suppression subtractive hybridization (SSH) and degenerate oligonucleotide primers to amplify cDNA of differentially expressed genes and nucleotide-binding site (NBS)-containing sequences respectively. This served as a source for the identification and characterization of disease-resistance genes in the wheat line PI 294994 after infestation with the Russian wheat aphid. SSH is an effective approach for studying the genetic nature of many biological processes by identifying differentially expressed genes. The sequences that were obtained in this study did indeed show some similarity to the underlying mechanisms involved in lesion formation and disease resistance. At the amino acid level similarities were identified with polypeptides such as lymphoma receptors. At the nucleotide level similarities were identified to enzymes involved in fibrinogen lysis and the chloroplast regulatory system. A heat shock protein gene was also identified, which may possibly play a role in the induction of the resistance response during aphid infestation. The use of PCR with degenerate oligonucleotide primers, designed from the NBS region of cloned disease resistance genes, has led to the cloning of resistance sequences in various plant species. In this study oligonucleotide primers designed from conserved motifs in the NBS domain were used to clone disease resistance gene homologues from the wheat line PI 294994. The sequences obtained showed a homology to an NBS region in lettuce, which was linked to a resistance protein candidate gene. In the wheat line PI 294994 the cloned NBS sequence was expressed at varying levels in different wheat tissues, which requires further study. However, when the full-length cDNA sequence of the NBS-containing gene was sequenced, there was no significant homology to any disease resistance genes at the nucleotide level. At the amino acid level there was homology to a kinase type resistance protein. The degenerate NBS

PCR approach was very successful in producing a number of NBS regions, which may be used for further study even if they are not necessarily involved in disease resistance. These results have aided the process of identifying novel aphid induced transcripts after infestation and has contributed to the growing base of knowledge about the underlying mechanisms involved in lesion formation and resistance responses to insects.

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Opsomming

Sedert die eerste waarneming van Russiese koringluis (*Diuraphis noxia* Mordvilko) in Suid Afrika in 1974, is gootskaalse verliese by klein-graan gewasse gerapporteer. Dit is ook die geval vir ander lande. Die ernstige skade wat deur *D. noxia* op koring (*Triticum aestivum*) en gort (*Hordeum vulgare*) veroorsaak word, het tot 'n gesamentlike poging deur wetenskaplikes gelei om die meganismes en gene betrokke by die weerstandsreaksie te ondersoek. Die poging lei tot 'n beter begrip van die meganisme en gene betrokke by die RWA weerstandsreaksie wat uiteindelik gebruik kan word om nog meer luisweerstandbiedende gewasse te kweek. Die doelwit van die huidige studie was om subtraksie-supressie-hibridisasie (SSH) en gedegenereerde oligonukleotiedvoorvoerders te gebruik om komplimentêre-DNA (cDNA) van differensieël uitgedrukte gene en nukleotiedbindingsetels- (NBS) -bevattende volgordes onderskeidelik te amplifiseer. Dit is gedoen om sielteweerstandsgene in die koringlyn PI 294994, na infestering met die Russiese koringluis, te identifiseer en te karakteriseer. SSH is 'n effektiewe nadering om die genetiese aard van baie biologiese prosesse te bestudeer deur die identifikasie van differensieël uitgedrukte gene. Die volgordes wat in hierdie studie verkry was, het wel sommige ooreenkomste met die onderliggende meganismes gewys en was dan wel funksioneel in letselformasie en siekteweerstand. Op aminosuurvlak is ooreenkomste geïdentifiseer met onder andere limfoomreseptors. Terwyl ooreenkomste met ensieme wat by fibrinogeenoplossing en die chloroplasreguleeringssisteem betrokke is, op nukleotiedvlak geïdentifiseer is. 'n Hitte-skok-proteïen is ook geïdentifiseer wat dalk 'n rol in die induksie van die weerstandsreaksie gedurende plantluisinfestering mag speel. Die gebruik van die polimerasekettingreaksie (PCR) met gedegenereerde oligonukleotiedvoorvoerders van die NBS-gebied, het tot die klonering van weerstandvolgordes in verskeie plantsoorte gelei. In dié studie is oligonukleotiedvoorvoerders vanaf gekonserveerde motiewe in die NBS-gebied ontwerp en gebruik om die siekteweerstandgeen-homoloë van die koringlyn PI 294994 te kloneer. Die volgordes wat verkry is, het homologie met 'n NBS-gebied in blaarslaai getoon wat aan 'n weerstandproteïenkandidaatgeen gekoppel is. In die koringlyn PI 294994 is die gekloneerde NBS-volgorde in variërende vlakke, in verskillende koringweefsel uitgedruk. Hierdie aspek regverdig verdere studie. Die volledige cDNA-basisvolgorde van die NBS-bevattendegeen het geen betekenisvolle

homologie aan enige siekteweerstandsgene op nukleotiedvlak getoon nie. Op aminosuurvlak was daar wel homologie met 'n kinase-tipe weerstandsproteïen. Die gedegenereerde NBS-PCR-nadering was baie suksesvol om 'n aantal NBS-bevattende fragmente te produseer, al hou dit nie noodwendig met siekteweerstand verband nie. Hierdie fragment kan vir verdere studie gebruik word. Hierdie resultate het die proses van identifikasie van nuwe plantluis-geïnduseerde transkripte na infestasie geondersteun en bygedra tot die groeiende basis van kennis oor die onderliggende meganismes wat by letselformasie en die weerstandsreaksie teen insekte betrokke is.