MOLECULAR CHARACTERIZATION OF GENETIC VARIATION IN *S. GRAMINICOLA* AND UNDERSTANDING HOST PATHOGEN INTERACTION

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CERTIFICATE

Certified that the work incorporated in this thesis entitled, "**Molecular characterization of genetic variation in *S. graminicola* and understanding host pathogen interaction**" submitted by Ms. Renuka S. Singru was carried out by the candidate under my supervision. The material obtained from other sources has been duly acknowledged in this thesis.

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Research Guide

Declaration

I hereby declare that the thesis entitled "**Molecular characterization of genetic variation in *S. graminicola* and understanding host pathogen interaction**" submitted for PhD degree to the University of Pune has not been submitted by me to any other university for a degree or diploma.

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List of Abbreviations

AFLP	:	Amplified Fragment Length Polymorphism
avr	:	Gene for avirulence in plants
bp	:	Base Pair
°C	:	Degree Celsius
cm	:	Centimetre
CTAB	:	Hexadecyltrimethyl Ammonium Bromide
cv	:	Cultivar
DNA	:	Deoxy Ribonucleic Acid
dNTP	:	Deoxy Ribonucleic Tri Phosphate
EDTA	:	Ethylene Diamine Tetra Acetic Acid
g	:	Centrifugal force based on gravitation
gm	:	Gram
h	:	Hour (s)
ha	:	Hectare
HCl	:	Hydrochloric acid
ICRISAT	:	International Crop Research Institute for Semi-Arid Tropics
ISSR	:	Inter Simple Sequence Repeat
kb	:	Kilobase pair
kg	:	Kilogram
lt	:	Liter
LTR	:	Long Terminal Repeat(s)
min	:	Minute
M	:	Molar
MAS	:	Marker Assisted Selection
Mb	:	Megabase pair
mm	:	Millimetre
mM	:	Millimolar
Mt	:	Million tonnes
MW	:	Molecular Weight
ng	:	Nanogram
nM	:	Nanomoles
ORF	:	Open Reading Frame
P	:	Phosphorous
PAGE	:	Poly-Acryl amide Gel Electrophoresis
PCR	:	Polymerase chain reaction
pH	:	Chemical reaction based on presence of hydrogen and hydroxy ions indicating acidity, neutrality or alkalinity
pmoles	:	Picomoles
p.s.i.	:	Pounds per square inch
PVP	:	Polyvinyl pyrrolidone
QTL	:	Quantitative Trait Loci
RAPD	:	Random Amplified polymorphic DNA
RFLP	:	Random Fragment Length Polymorphism
RIL	:	Recombinant Inbred Line
rpm	:	Revolutions per minute
RT	:	Retrotransposon
s	:	Second (s)
SCAR	:	Sequence Characterized Amplified Region

Sg	:	*Sclerospora graminicola*
SSC	:	Trisodium citrate
SSR	:	Simple Sequence Repeat
STMS	:	Sequence Tagged Microsatellite Site
STS	:	Sequence Tagged Site
TAE	:	Tris Acetate EDTA
TE	:	Tris EDTA
TE	:	Transposable element
Tris	:	Tris-hydroxamethyl amino methane
U	:	Unit
µg	:	Micro gram
µl	:	Micro litre
µM	:	Micro molar

Summary

Pearl millet (*Pennisetum glaucum* (L.) R.Br.) is of critical value for food security in some of the world's hottest and driest cultivated areas of Africa and Asia. Downy mildew, incited by *Sclerospora graminicola* (Sacc.) Schroet. is the most widespread disease of pearl millet (Singh et al., 1993). *S. graminicola* is an oosporic, systemically infecting heterothallic fungus affecting the crop at the seedling stage and transforming the panicle into a malformed ear head, called green ear. The losses can be very high under favorable conditions of disease development in a susceptible cultivar. Studies on this oomycete pathogen are complicated by the fact that it cannot be grown as an axenic culture in laboratory. The introduction of potentially high yielding hybrids into India in the late 1960s led to a large-scale cultivation of homogenetic material. When the first of several downy mildew epidemics occurred in 1970-71 (Andrews et al., 1985), there were three consequences of resistance breakdown: withdrawal of several hybrids, yield reductions and an increase in pathogen inoculum. Resistance is known to be regionally variable, and, therefore, new breeding material has to be tested in expensive multi-location trials (Ball et al., 1986).

I endeavored to study the downy mildew population based on molecular variations and to solve some queries on the host-pathogen interaction using amplified fragment length polymorphism (AFLP) as a molecular tool to resolve genetic differences among 19 isolates of downy mildew from different geographic locations where pearl millet is grown. Based on the marker data, unweighted pair-group methods of averages cluster analysis and principal coordinate analysis separated the mildew collections into 4 distinct groups. Pathogen isolates having opposite mating ability as (+) and (−) strains to produce sexual oospores confirmed the genetic relationship to expected variation. In some cases, the genetic distance between the isolates reflected the physical distance between collection sites. The use of AFLP as a DNA fingerprinting method to detect genetic variation would be particularly important in selecting mildew isolates in screening breeding material for identification of resistance and monitoring genetic changes in *S. graminicola* populations in relation to changes in host cultivar.

The downy mildew pathogen is capable of infecting a previously resistant host as it evolves rapidly to new virulent forms, a characteristic of retrotransposon activation. Retrotransposons are mobile repeat elements, and are known to mutate the organism's genes on activation by abiotic and biotic stress factors. I screened the λgt11 phage genomic

library of Path-6 isolate of *S. graminicola* with P5 retro element that originated from another fungal pathogen. Some analogues were isolated and their distribution among 5 diverse host specific pathotypes was studied. In this investigation, I also tried to study the role of P5 in compatible and incompatible pear millet-downy mildew interactions in nature.

A DDRT - PCR (Differential Display Reverse Transcriptase PCR) approach was implemented to unravel the underlying mechanisms in the process of infection and the interplay of signaling between host and pathogen. The main advantage of this technique is it permits the simultaneous identification of up and down regulated genes. DDRT - PCR has been used to isolate mRNA species in plants induced in response to biotic and abiotic stress (Liang and Pardee, 1992). The DDRT study could show differential expression between healthy and infected pearl millet seedlings. I also tried to elucidate the role of certain defense-related genes namely PAL (Phenylalanine ammonia lyase), lipoxygenase, chitinase in resistance of pearl millet to downy mildew. The changes in the levels of these defense proteins were found to be associated with incompatible and compatible interactions. An attempt was made to correlate temporal patterns of morphological events of the infection process.

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Chapter 1

General Introduction

Diseases are one of the major constraints in the cultivation of crops especially in today's monocropping environment that becomes conducive for the multiplication of plant pathogens. Among various phytopathogens, the fungi have been historically and are currently the most important pathogens of crops causing far-reaching damages all over the world. My thesis has focused on specific molecular aspects of interaction between pearl millet, (*Pennisetum glaucum* (L.) R.Br) and its fungal parasite downy mildew, (*Sclerospora graminicola*). There are three sections in this introduction chapter; in Section 1.1, I have reviewed various crop diseases caused by fungi and their impact on agriculture and the conventional and biotechnological strategies to control some of these diseases. In Section 1.2, I have attempted to summarize the information about downy mildew of pearl millet and Section 1.3 portrays the genesis of my work.

1.1 Fungal Diseases: an important factor affecting crop productivity

The significance of a particular outbreak of fungal disease is based on its magnitude and the events leading to the losses can be understood by conducting virulence studies. Some examples of the major classes of fungal pathogens and their interacting hosts include lettuce-*Bremia lactucae*, tobacco-*Phytophthora parasitica*, grass species-*Magnaportha grisea*, tomato-*Cladosporium fulvum* and barley-*Rhyncosporium secalis* (Crute, 1985; de Wit, 1995; Hulbert and Michelmore, 1988).

In the Indian context, several cases illustrate how fungal diseases affect crop productivity. For example, foliar blights and scab of wheat have been intensified due to the rice-wheat cropping system in north-west India resulting in outbreaks where up to 36 % yield has been lost (Singh and Srivastava, 1997). Powdery mildew, which occurs in North Western parts of India and hilly regions, attacks both wheat and barley and causes enhanced losses ranging between 5-50 %. *Rhyncosporium secalis*, an imperfect fungus, is the causal agent of barley leaf scald, killing the host cells as a source of nutrients and causing losses in several parts of the world. The filamentous heterothallic ascomycete, *Magnaporthe grisea*, causes blast disease on more than 50 different graminaceous species including rice, finger millet and weeping love grass. Sheath blight, one of the potentially serious diseases caused by *Rhizoctonia solani* Kuhn that reduces the grain yield of rice, maize and other cereals, has been reported in many countries and causes yield losses to the extent of 11-97 % (Saxena, 1997). In India, this disease is found to occur in Uttar Pradesh, Kerala, Andhra Pradesh, Kashmir and Tamil Nadu (Laha et al., 2000). Blast, caused by *Pyrcularia grisea*, is a major disease of rice as it causes serious damage in upland as well as irrigated regions

under high fertility levels and congenial environmental conditions. Foliar diseases like downy mildews and leaf rusts have caused extensive economical damage to maize in South East Asia where the area is under maize-rice rotation, thereby affecting valuable tropical and sub-tropical germplasm (Saxena, 1997). Grain mold is the most important disease of sorghum incited by non-specialized fungi of several genera including *Fusarium*, *Curvularia* and *Phoma*. It is present in all sorghum-growing areas of the world where it reduces both quality and quantity in the range of 30-100 % of the crop (Singh and Thakur, 1995).

(i) Conventional strategies to control fungal attack on crops

The high rate of growth in the 1980s world cereal production has been attributed to the worldwide adoption of the Green Revolution. The latter has marked the widespread use of specific disease resistant cultivars and cultural practices that enhanced the crop productivity. The availability of resistant cultivars, together with fungicides, cultural practices and biological control methods have provided opportunities for effective management of the fungal diseases and the brief information of each approach is as follows:

Host-Plant Resistance: Host plant resistance has always been among the most important biologically based methods of plant disease management. Factors that influence resistance expression in plants are: genetic nature of resistance, cropping systems, variability in pathogen population, and weather factors. In grain mold of sorghum, three mechanisms of resistance – grain hardness, lax panicles and glume coverage - have been combined in agronomical elite cultivars to provide resistance. Host plant resistance is less stable in cases where the pathogen is highly variable. For example, soil borne pathogens are normally less variable than air-borne pathogens, and pathogens, which reproduce asexually, are less variable than those that reproduce sexually. Similarly, weather conditions that are favorable for pathogen survival and disease spread, influence host growth and resistance expression, and the resistance does not hold under high inoculum pressure (Thakur, 2000). Success also depends on the genetic differences among the cultivars planted in given areas. Open pollinated varieties are highly heterogeneous, though not disease free, since they do not develop the disease in epidemic proportions for several years. Wild species are another source for improvement of host resistance. Using the rice-wheat rotation as an example of host resistance measure, increased emphasis is placed on developing cultivars adapted to adverse conditions such as heat and cold tolerance,

earliness, tropical diseases, water logging resistance and also to drought tolerance where there are distinct wet and dry seasons (Khush and Baenziger, 1998).

Fungicides: Several cases have proved that a combination of fungicides is required for successful disease control. For example, wheat seed is treated with difenconzole plus metalaxyl for protection against infection of germinating seeds and young seedlings from *Rhizoctonia* and *Pythium* species respectively (Cook, 1998). This early protection improves seedling vigor, which can also set the final yield potential. Seed treatment with Triademinol and treatment with systemic fungicides reduces the extent of powdery mildew of wheat in many environments (Everts and Leath, 1993; Melikova, 1991). In maize, single spray application of Thiobendazole has reduced the banded sclerotial disease effectively and has led to a significant effect on yield parameters such as grain weight, average number of cob per plant and grain yield (Lal et al., 1985). Another observation is that the efficacy of fungicides is dependent on host cultivar (Sinha, 1992).

Cultural practices: Management of disease can be done by adjusting of sowing time, deep ploughing to escape soil borne diseases, intercropping, removal of collateral hosts and strategically cleaning seeds. It has been found that location specific sowing schedule of maize could help the crop to escape disease caused by *Claviceps* (Virk et al., 1981). Soybean crop is sown at such a time that the peak humidity during reproductive phase is avoided (Thapliyal and Singh, 1997). Deep ploughing helps to bury the sclerotia deep in to the soil reducing the soil surface primary inoculum. Intercropping of maize with legumes especially with soybean reduced pathogen activity in soil (Lehman et al., 1976). Lee et al. (1989) have found that by maintaining the maize population at an appropriate level and applying cattle compost before planting decreases the disease level and subsequent spread of *Rhizoctonia solani* in field. Small scale cleaning sclerotia from some cereal seeds can be done using 10 % common salt (Nene and Singh, 1976) while large scale cleaning can be done by various mechanical separators (Chahal et al., 1994). Lower planting densities and improved spacing promote better aeration of foliage and is less conducive to disease.

Biological control: The use of plant-associated microorganisms has shown some potential for management of several important plant diseases especially those caused by bacteria (Kerr, 1980) and fungi (Chen et al., 1996). Success depends on the ability of the introduced strain to: (a) colonize the rhizosphere, (b) produce an antibiotic, enzyme, and/or elicitor, (c) display systemic acquired resistance to prevent infection or (d) suppress disease development. Several projects in Asia are testing an aerosol spray method for application

of bacteria to foliage for biological control of rice sheath blight caused by *Rhizotonia solani* AG1 and the results have been encouraging (Chen et al., 1996). Research in Australia has demonstrated the potential for the use of antibiotic-producing bacteria introduced on the seeds of wheat to manage root diseases, particularly the take-all disease (Cook, 1993). Extensive work on soil application of *Trichoderma* and *Gliocladium* preparations has documented that fungi can be effectively controlled for several seed rot and seedling diseases of groundnut, chickpea and maize crops (Mukopadhyay, 1996).

(ii) Modern biotechnological approaches to control fungal pathogens

In recent years, promising protocols for transformation and regeneration of crops have been established which enabled the introduction of single genes for disease and insect resistance (Shimamoto et al., 1989; Christau et al., 1991). The technology allows the transfer of resistant traits into plants without altering their intrinsic properties. Emerging strategies to control fungal diseases pursue two approaches. The first one focuses on the production of antifungal compounds in transgenic plants that directly affect the fungus, i.e. antifungal proteins and toxins. For example, genes coding for chitinase have been introduced into rice (Lin et al., 1995) where they impart resistance to sheath blight, a serious fungal disease. The second approach aims at the generation of plant responses leading to cell death upon pathogen infection. Systemic acquired resistance in susceptible plants to virulent pathogens has been achieved by the increased expression of chitinase by the introduction of a chitinase transgene (Broglie et al., 1991). There is also the prospect of combining transgenes for production of chitinase, β 1,3-glucanase as cell wall degrading, pathogenesis related (PR) genes and/or ribosome-inactivating protein which inhibits growth by inactivating ribosomes of foreign species like those from fungi, to confer a greater level of resistance (Jach et al., 1995). Another group of antifungal proteins is the small cystein-rich proteins called defensins, for example, Rs-AFP2 is an antifungal protein from *Raphanus sativus* in transgenic tobacco plants which showed enhanced resistance to *Alternaria longipes* (Broekaert et al., 1995).

The first report on transgenic plants constitutively producing a single pathogenesis related (PR) protein with *in vitro* antifungal activity has been on a tobacco PR3 protein (class I chitinase). These plants however have not shown a significant difference in susceptibility to the pathogen, *Cercospora nicotianae* (Neuhaus et al., 1991) suggesting that class I chitinase alone is unable to bring about resistance *in planta*. Since then a number of reports dealing with fungal resistance of transgenic plants constitutively expressing chitinase or

glucanase classes of PR genes have been released, with enhanced resistance in many cases. Recently, a number of reports have appeared in which constitutive co-expression of PR2 and PR3 genes has resulted in an increase in resistance against several fungi, which explains synergy between the two gene products and between antifungal proteins both *in vitro* and *in planta* (Kumar and Ziegler, 2000; Cook 1998; Khush and Baenziger, 1998). Though most tests have been performed under greenhouse conditions, reports of successful field trials with plants containing single PR gene products have been notably a hybrid endo-chitinase in canola (Grison et al., 1996) and a tobacco PR5 gene in carrot (Stuiver et al., 1996). Plants can also be engineered to produce antibodies to inactivate the molecules that are necessary for pathogens to successfully infect plants. For example, when the hen egg white lysozyme (HEWL) is expressed in transgenic potato and tobacco plants, antimicrobial activities to several bacteria and fungi like *Botrytis cinerea*, *Verticillium albo-atrum* and *Rhizotonia solani* are observed. However, with this biotechnology approach, fungi containing mainly chitosan or cellulose in their cell wall are not inhibited in their growth. Engineering plants to produce phytoalexins upon fungal infection is one of the strategies used to obtain fungal resistance, as these compounds are produced in plants after pathogen attack and abiotic stress. Interfering in the phytoalexin synthesis has been difficult as the pathways are complex. Studies on tobacco-*Botrytis cinerea* reported that engineering the plant stilbene synthase gene for production of a stilbene-type of phytoalexin resulted in increased resistance to the fungal pathogen (Khush and Baenziger, 1998).

(iii) Molecular breeding of crops for sustainable resistance against fungal diseases

Plant breeding has three phases namely introduction of genetic variability, selection of useful recombinants, and evaluation of selected recombinants in diverse environments to identify new cultivars and their area of adaptation. Where rare, specific traits are needed, the use of wild relatives (Jiang et al., 1994) in backcrossing programs and genetic engineering (Dale et al., 1993) will become increasingly important as they increase genetic variability for selection. Diversification and broadening of the genetic base of crops is being pursued by introgression of genes from primary, secondary and tertiary gene pools to achieve multi-stress resistances, hybrid vigor, physiologically efficient plant types and enhancement of yield potential. Of course the success is dependent on availability of a suitable recurrent parent or recipient line for the transferred gene(s). These lines are invariably developed from the progeny of elite crosses. Use of selection nurseries that magnify the differences among genotypes in a breeding program is another approach. In

this aspect, there has been a trend towards mapping genes of interest in the crops with goal of tagging the genes for eventual use in Marker Aided Selection (MAS). Detailed maps of related crops provide a valuable tool for the location of important genes and become more applicable as the density of the maps improves. In rice, QTLs for blast and bacterial blight resistance have been tagged and pyramided into an improved varietal background through MAS). In case of wheat, tagging of genes for disease and herbicide resistance are underway to maximize wheat rotational systems (Reide and Anderson, 1996). Markers linked to *Fusarium* wilt resistance in chickpea are also available for use in MAS.

Numerous breeding lines with desired characteristics have been developed and are being evaluated in replicated yield trials (Khush and Baenziger, 1998). In rice, many crosses between elite breeding lines and wild species have been accomplished through embryo rescue technique. Similarly, genes for disease resistance have been transferred from several wild species into cultivated rice (Jena and Khush, 1990).

Another strategy to increase yield potential is the exploitation of heterosis. Rice hybrids with a yield advantage of about 10-15 % over inbred varieties have been introduced in China in the mid-1970s and are now planted to 45 % of the riceland in that country. Rice hybrids adapted to tropics have now been planted in India and show a similar yield advantage. It has been found that the magnitude of heterosis depends upon the genetic diversity between the parents of the hybrids.

Presently, the complete evaluation of a crops' genome for agronomic performance requires a tremendous effort (laboratory, field and statistical) that only a few crosses (far too few for a breeding program) can be thoroughly analyzed (Tinker et al., 1996). However, it is certain that all approaches, including conventional as well as biotechnological will be utilized to increase the resistance and yield potentials of crops.

1.2 Pearl Millet – Downy Mildew System

(i) The host pearl millet, *Pennisetum glaucum* (L.) R. Br.

The common names of pearl millet are bulrush millet, bajra and bajri (King and Thakur, 1995) and Figures 1 and 2 represent a typical healthy crop. It is an important crop providing staple diet for people living in large areas of the semi arid tropics in Africa, Middle East and South East Asia and as a forage crop in the USA.

Figure 1 Healthy panicles of pearl millet, photos taken at ICRISAT, Patancheru, India.

In terms of annual global production, it is the sixth most important cereal crop, after wheat, rice, maize, barley and sorghum (FAO, 1992). Pearl millet and other millets are more important than statistics indicate because in areas where they are grown for food, these are the only crops people can grow as a source of energy and protein. Pearl millet can grow in a wide range of agro-climatic conditions, ranging from the tropics to hot areas of temperate zones. Its capacity to grow and yield reasonably well on sandy, marginal soils, or in soils of low pH, high salinity or low fertility, makes it a staple food crop in many low input, drought prone, agricultural areas of African and Asian countries. In addition, under unfavorable environmental conditions it has the potential to perform better than other cereals, such as wheat, rice, maize or sorghum, with respect to grain and fodder yield (Govila, 1993). It is grown primarily by subsistence farmers in marginal areas where the annual rainfall varies between 250 mm to 800 mm. Nutritionally superior to rice and wheat, pearl millet is commonly baked as unleavened bread, or cooked as thin or thick porridge.

Figure 2 Pearl millet field with healthy hybrids at ICRISAT, Patancheru, India.

In India, pearl millet is the principal grain and fodder crop in Rajasthan accounting for 45% of total Indian crop. Other states where this crop is also grown are Maharashtra, Gujarat, Uttar Pradesh, Haryana, Andhra Pradesh, Karnataka and Tamil Nadu (Indian Agricultural Statistics, 1973-74). In Africa, the pearl millet crop is of most importance in the Sahel where each of the seven countries in this region grows about one million ha or more pearl millet annually. Table 1 shows the production of pearl millet in the world and different continents.

Table 1 Worldwide production of pearl millet in 1994 (FAO, 1995).

Crop	Region	Area (x 1000ha)	Yield (kg ha^{-1})	Production (x 1000MT)
Pearl millet	World	37 710	689	25 982
	Africa	18 214	591	10 758
	Asia	17 709	800	14 169
	South America	37	1432	53
	USA	150	1200	180

(ii) The Pathogen downy mildew, *Sclerospora graminicola* causing downy mildew in pearl millet.

The four major diseases that cause economic losses to pearl millet are downy mildew (*Sclerospora graminicola*), ergot (*Claviceps fusiformis*), smut (*Tolysporium penicillariae*) and rust (*Puccinia substriata var. indica*). Among these, downy mildew is more widespread and devastating than others and has caused losses in the range of 20-80 %. Compared to rusts and powdery mildews, research on this fungus is limited due to difficulties faced in maintaining it on living host plants, long term storage of viable sporangial inoculum, infection with oospores, diploid status of the mycelium and spores, recovery of progeny from oospores and heterothallism (a phenomenon in which sexual multiplication of the downy mildew takes place in presence of two mating types).

Sclerospora graminicola (Sacc.) Schroet can infect young tissue in plants causing downy mildew in the main shoots when plants are young; in tillers when the crop is older by means of sexual reproduction (oospores) and by asexual reproduction (sporangia and zoospores). Figure 3 shows a green ear of systemically infected pearl millet shoot while Figure 4 depicts advanced stages of necrosis with the browning and drying of the ear in complete plants of pearl millet in field. Once a shoot or tiller is infected, no grain will be produced because of the inflorescence phyllody that has given this disease its local name ‘green ear‘ (Ball and Pike, 1983).

Figure 3 Downy mildew disease of pearl millet green ear with sterile inflorescence. Photo taken at ICRISAT, Patancheru, India.

Typical symptoms of downy mildew infection on pearl millet begin with chlorosis at the base of the leaf, called 'partial leaf' symptom (Williams, 1984), which gradually covers a greater number of leaves until the entire leaf is chlorotic. Asexual sporulation occurs in favorable conditions on the abaxial side of infected leaves, leaving a fine white powder of spores, hence the name downy mildew. Systemically affected pearl millet plants bear no panicles, instead produce leafy growths as depicted in Figure 3. Eventually, the green ear becomes brown and full of oospores of the pathogen as shown in Figure 4.

Figure 4 Downy mildew diseased plants in ICRISAT fields, Patancheru, India.

The disease cycle of downy mildew on pearl millet includes a sexual and an asexual phase as depicted in Figure 5. The seed or soil borne inoculum acts as a primary inoculum source to initiate the expression of the disease in a host population, resulting in infection of seedlings. Severely infected young seedlings mostly die within 30 d without producing oospores. Plants which express disease symptoms about 20 d after sowing due to soil borne

oospores, seed borne inoculum, or secondary infection by airborne sporangia (Reddy, 1973; Subramanya et al., 1982), produce millions of sporangia during earlier growth phases.

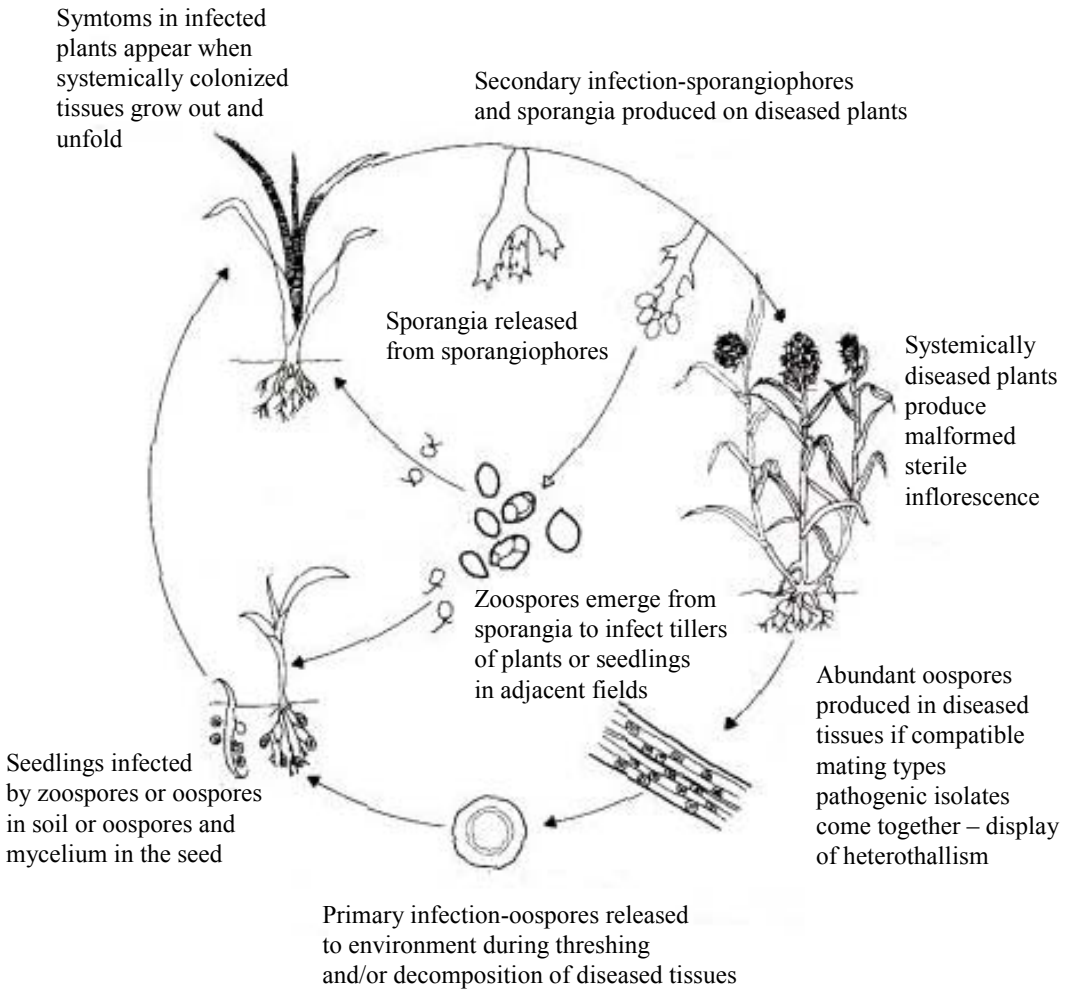

Figure 5 Disease cycle of downy mildew taken from the following reference with some changes: Shetty HS (1987) Biology and epidemiology of downy mildew of pearl millet. In: *Proceedings of International Pearl millet Workshop*, 1986. Patancheru, India: ICRISAT, pp 147-60.

(iii) *Sclerospora graminicola* pathogen alters rapidly and dramatically

The introduction of potentially high yielding hybrids into India in the late 1960s led to mass planting of homogenetic material over large areas. Reportedly, these hybrids have a common, downy mildew susceptible, female parent that has been introduced from Tifton,

Georgia, USA where downy mildew is of no relevance (Murty et al., 1983). By 1971, over 2 million ha out of a total of 12 million ha had been planted as hybrids when the first of several downy mildew epidemics occurred (Andrews et al., 1985). At this time, neither fungicides nor effective resistant cultivars were available (Singh et al., 1997). Unfortunately, the susceptible hybrids continued to be cultivated despite their known susceptibility to downy mildew (Pokriyal et al., 1976). As a result, there have been three consequences of resistance breakdown: withdrawal of several hybrids, yield reductions and an increase in pathogen inoculum. In India, a major epidemic occurred in 1971-76, the resultant oosporic build-up in the fields posed a major threat to the survival and continuation of local and hybrids, which were previously considered highly resistant (Singh et al., 1986). Losses of 10-60 % have also been reported in African countries such as Africa, Nigeria (King and Webster, 1970), and Tanzania (Doggett, 1970). *S. graminicola* is a highly variable pathogen and exhibits geographic variability (Ball et al., 1986, Singru et al., in press). Great heterogeneity is found within populations between seasons and single oospore isolates in India (Thakur and Shetty, 1993) and Africa (Ball, 1983) suggesting that the pathogen has potential to alter rapidly and dramatically.

Changes in downy mildew isolates in India and Africa, from the 1970s have been characterized phenotypically (Ball, 1983 and Thakur, 1992) and there has been only one report (and one in press) related to genetic differences based on fingerprinting data using mini- and micro-satellites (Sastry et al., 1995). The molecular data of pathotyping is considered to be more precise and reliable than biological pathotyping, as it is not influenced by environmental factors (Jeger et al., 1998). The genetic diversity in pathogen population is an important consideration in planning disease management strategies. Today, ICRISAT conducts regular surveys to assess the impact of downy mildew on pearl millet through most of the pearl millet growing regions of India (Rao et al., 2000). There are several examples of occurrence of variable pathotypes in India where about 40% of the pearl millet crop is sown as hybrids (Singh et al., 1993). These pathotypes have been detected on the basis of differential reactions using hybrids and inbreds (King et al., 1989; Thakur and Shetty, 1993). Michelmore et al. (1982) have reported that *S. graminicola* is heterothallic with two compatible mating types, namely PT 2 and PT 3. Idris and Ball (1984) have confirmed this by investigating some isolates from India and Africa. The high genetic variability leads to rapid selection and adaptation to new host genotypes, as demonstrated by selection of asexual generations from a field population developed for host genotype specific virulence (Thakur et al., 1992, Sastry et al., 2001). Data by Thakur

and Rao (personal communication) indicates that differential host-pathogen interactions and distinct groupings of pathotypes and genotypes occur. High genetic variability for virulence accounts for the ability of the pathogen population to adapt to new, uniform host genotypes, such as F1 hybrids (Singh et al., 1993). When such constraints are put on the system, features usually present in minor quantities emerge as a result of selection pressure. The alterations in frequencies of particular elements within the pathogen populations could have been responsible for intercontinental differences between Indian and African spore collections recorded (Ball et al., 1986). Differences in nuclei number and size of sporangia in two different pathogenic races have been reported (Shetty and Ahmed, 1981).

(iv) Geographic distribution and economic importance of *S. graminicola*

Although downy mildew is prevalent in temperate and tropical areas of the world, including the Middle East, Africa, and Asia (Safeeulla et al., 1976), it has been originally reported as being a problem only in low lying, poorly drained areas where heterogeneous land races were being cultivated and so the disease attracted little attention (Butler, 1918). Today, *S. graminicola* is found in most tropical and subtropical regions where pearl millet is cultivated (Williams, 1984). It is found in countries namely Burkina Faso, India, Malawi, Mozambique, Niger, Nigeria, Senegal, Tanzania, Zambia and Zimbabwe where pearl millet is grown on a commercial scale (Chahal et al., 1994). The causal organism of this disease has been reported on different graminaceous hosts in other countries such as China, Fiji, France, Germany, Hungary, Iran, Israel, Italy, Japan, Netherlands, Romania, Russia, Spain, and USA (Safeeulla, 1976). The pathogen also infects maize and sorghum, and is found in the 51 countries represented in Table 2.

In India, *S. graminicola* has been first reported on pearl millet by Butler in 1907 but downy mildew did not become a serious problem until after the widespread cultivation of hybrids in late 1960s. Yield losses from 6 to 60% have been reported from India, Mozambique, Nigeria and Tanzania (Doggett, 1970; King and Webster, 1970; Mathur and Dalela, 1971; AICMIP, 1971). Between 1962-1964, the monetary loss due to downy mildew in Rajasthan, India is reported to be 20 million rupees each year (Safeeulla, 1976). India has suffered up to 60% losses on the hybrids during the epidemic years (AICMIP, 1972-87). The most popular hybrid in 1973, HB-3, devastated one million ha in India causing 10-45% loss in various localities. In 1983, BJ 104 losses caused the same magnitude of loss (Thakur et al., 1992). This disease affects the grain yield, extensive damage to vegetative

parts of the plants, making the infected crop unfit for use as fodder. In severe cases, it has wiped out the entire crop causing complete loss.

Table 2 Countries from which *S. graminicola* has been reported (Jeger et al., 1998).

Region or continent	Country
Africa	Burkina Faso, Chad, Egypt, Ethiopia, Gambia, Ghana, Ivory Coast, Malati, Mali (N'Diaye, 1995), Mauritiana (Frison and Sadio, 1987), Mozambique, Niger, Nigeria, Senegal, Sierra Leone, South Africa, Sudan, Tanzania, Togo, Uganda, Zimbabwe
Asia	China, Hong Kong, India, Iran, Israel, Japan, Kazakhstan, Korea, Pakistan, Taiwan, Yemen
Oceania	Fiji, Hawaii
Europe	Bulgaria, Caucasus, Czech Republic, France, Hungary, Italy, Netherlands, Poland, Rumania, Spain, Switzerland, Yugoslavia
North America	Canada, Mexico, USA
Central America	Puerto Rico,
South America	Argentina
Unless otherwise indicated, all reports were obtained from CMI Distribution Maps of Plant Diseases, Map no. 341, Edition 2, Issued 1 October 1979.	

(v) Measures employed to control downy mildew

Host Plant Resistance: Genetics and inheritance of resistance are better understood for downy mildew (Singh and Talukdar, 1998) than for other diseases of pearl millet. Resistance to pearl millet downy mildew is reported to be dominant over susceptibility and is probably controlled by few genes (Thakur et al., 1992; Singh, 1995). Use of homozygous sources of resistance and susceptibility combined with precise infection techniques are underway to study genetics and inheritance of resistance while recovery resistance may be applicable in certain cases (Singh and King, 1988). Induced resistance, whereby a plant becomes resistant by pre-exposure to a low level inoculum may have a practical value (Kumar and Andrews, 1993). Landrace improvement (including local varieties) is an important option for improved cultivars of pearl millet. Introducing elite germplasm can enrich genetic variation of pearl millet landraces or germplasm from other

landraces growing under conditions similar to those of the target area. On the other hand, commercial production of pearl millet is made possible by good cytoplasmic male sterility (CMS) systems. Gene deployment can also be used through reduction of pathogenicity if the host specific to the oospore population is withdrawn over time of cultivation. The development of DNA markers has enabled genes contributing towards complex traits such as disease resistance and tolerance to adverse abiotic conditions, to be mapped using quantitative trait locus (QTL) analysis (Hash et al., 1997). In pearl millet, gene pyramiding is being implemented to obtain downy mildew resistant cultivars in combination with drought tolerance though transgenesis has not been attempted so far.

Chemical control: Metalaxyl as a foliar spray fungicide can still be used effectively for downy mildew disease control if the method of application is carefully worked out as the degree of control depends on the rate of application and level of varietal susceptibility. It is used to protect the pearl millet varieties in farmer's fields. Seed treatment is useful for commercial seed production and for small-scale farmers growing traditional and often susceptible cultivars. Combination of metalaxyl with other fungicides and with other management steps will ensure longevity of chemical control.

Cultural practices: Of the many cultural practices known such as crop rotation, early planting, seed sanitation, control of nitrogen, phosphorous and zinc levels in soils (Singh and Agarwal, 1979) and rouging, the latter is most recommended along with other control measures. Rouging has beneficial effects of reducing the source of spores within-season (asexual spores) and between seasons (sexual spores). It can also be applied to weed hosts to reduce the sources of external inoculum (Jeger et al., 1998). Interestingly, cultivation of a highly susceptible pearl millet variety between cropping seasons to control pearl millet downy mildew has been suggested (Thakur, 1992). The trap crop should be harvested as soon as the symptoms appear and before the production of oospores. However, this has been a limitation if the region has a short rainy season and low quality of farmland. Another important cultural practice followed is early sowing in semi-arid areas to avoid risks associated with lack of water and with insect pests. Plantation of 20-day-old seedlings has resulted in less downy mildew infection and 50% higher yields for both susceptible and resistant cultivars (Rao et al., 1987).

Biological control: Under experimental conditions oospores of *S. graminicola* were effectively killed by parasitism by *Fusarium semitectum* as reported by Rao and Pavgi (1976). In 1998, Umesha et al. reported the use of *Pseudomonas fluroscens* as

biological agent of downy mildew of pearl millet in greenhouse and field conditions. *P. fluroscens* was used as foliar spray and also in seed treatment. The combination of the two brought significant disease reduction. Also, the potential of *Trichoderma harazianum* and *Chaetomium globosum* has been demonstrated. It has been suggested to supply a formulation of *P. fluroscens* to farmers for sowing; further follow up studies are needed for the promotion of such a product.

(vi) Sources of resistance

In case of pearl millet, the first official release of a heterogeneous top-cross hybrid cultivar, Jawahar Bajra Hybrid-1 (JBH-1), has taken place in Madhya Pradesh, India (Chauhan GS, personal communication, 1996). Among the sources of complete resistance released in Africa (Singh et al., 1992), viz. IP 18292, IP 18293 etc., none has continued to be disease-free at all test sites and under all screening conditions. Some of the products of ICRISAT Pearl millet Improvement Program's recurrent selection project have been released for cultivation by national program in India, after they showed an ability to combine high yield stability and downy mildew resistance during several years of multi-locational trials (Williams and Andrews, 1983). These are WC-C75, ICTP 8203, ICMH 451 and Pusa 23 and male sterile line like ICMA 1 and ICMA 841 that are being grown over large areas in India. Several open–pollinated varieties are being grown in various countries in Africa. Some DNA markers have been identified for downy mildew resistance loci on several linkage groups with varying effects against different pathotypes of *S. graminicola* (Hash et al., 1997). Using DNA marker technology, downy mildew resistance genes are being transferred from P 7-3, P310-17, IP 18292 and some other elite breeding lines to develop hybrids with known resistance genes (Hash et al., 1997). A 1995 survey conducted in the pear millet growing areas at India has revealed that 37 cultivars (6 open–pollinated varieties, 30 single–cross hybrids and 1 top-cross hybrid) are being grown. Some cultivars, e.g., Pusa 3 are used extensively, while others have limited use, but all are being grown to serve as a 'cultivar mosaic' pattern in the field. This provides a high degree of heterogeneity in any given area.

Due to the heterogeneity in the pathosystem and the growth of genetically uniform single-cross F1 hybrids, namely the CMS Tift- 23 A from USA, a wide spectrum of pathogen virulence and host susceptibilities have occurred and this has to be avoided in future. Since 1995, though there have been reports of downy mildew occurrence in a few single–cross hybrids as these were cultivar–specific and field–specific (the same cultivar grown 1 km

away had no or little disease), epidemics have not taken place (Singh et al., 1997). High yield losses due to downy mildew seldom occur where heterogeneous landraces and improved open pollinated cultivars are grown. This is because the landrace varieties have developed genetic resistances, which operate best in the natural varietal state of a random mating population. Molecular techniques will be potentially useful in identification of resistant and superior genetic material and efforts are underway to tag resistance genes of these hybrids (Hash et al., 1997).

1.3 Focus of my thesis in context to pearl millet downy mildew interaction

In my thesis, I have focused my work on three aspects namely:

1) Understanding the genetic variability of the Indian pathogenic isolates of downy mildew, highlighting the basis of genetic changes rendered by the host on *S. graminicola*.

2) Revealing the presence of a retrotransposon, also known as repetitive DNA elements, in downy mildew, yielding a putative active element that is expressed in compatible host – pathogen interaction.

3) Unraveling the response of the host to infection, as determined by two approaches: (a) by northern analysis of vital defense-related RNAs and (b) by DDRT (differential display reverse transcriptase) for simultaneous identification of up and down regulated genes.

I used AFLP as a molecular tool to resolve genetic differences between 19 isolates of downy mildew from different geographic locations where pearl millet is grown. P5 retro element-like sequences were isolated from the λgt11 library of *S. graminicola* to study their distribution among 5 diverse host specific pathotypes. In this investigation, I also tried to study the role of P5 in compatible and incompatible pear millet-downy mildew interactions in nature. The DDRT -PCR (Differential Display Reverse Transcriptase PCR) approach was implemented to unravel the underlying mechanisms in the process of infection and the interplay of signaling between host and pathogen. I also tried to elucidate the role of certain defense-related genes namely PAL (Phenylalanine ammonia lyase), lipoxygenase, chitinase in resistance of pearl millet to downy mildew.

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Chapter 2

Detection of genetic variability in pearl millet downy mildew (*Sclerospora graminicola*) by AFLP

2.1 Introduction

The long-term effectiveness of disease resistance genes in cereal crops is dependent on the rate at which isolates with virulence to host resistance increases and becomes prevalent in the pathogen population. Genetic diversity and population structure of the pathogen influence the speed with which new types of virulence are selected and incorporated into pathogen population as a whole. In consequence, durability of disease resistance is likely to break down if the pathogen population is genetically diverse or if rates of mutations are high (Katiyar et al., 2000).

There are several examples of genetic heterogeneity among pathogens and its role in disease resistance. Researchers studying the genetics of soil-borne phytopathogenic fungus *Rhizotonia solani* have demonstrated the existence of mating types and heterokaryosis as a means of variation that resulted in infliction of high yield losses to vegetable and field crops, turf grass and ornamental fruit and forest trees (Julian et al., 1999). Similarly, variations at the genetic level have revealed heterogeneity between species and isolates of the complex genera *Colletotrichum*. Distinct lineages of *Pyricularia oryzae* and *Colletotrichum* which affect common bean are produced by asexual reproduction that cause differences in pathogenic variability at the genetic level which can be analyzed by molecular techniques (Gonzalez et al., 1998). Changes in *Puccinia graminis* races in wheat in Canada, from the start of the surveys in 1919 to the mid-1960s, have been characterized by a succession of races that originated from different asexual clusters. Factors such as introduction of single resistance genes into cultivars, highly related virulence capabilities of the isolates and greater frequencies of virulent isolates have been the causes for short-lived resistance in winter wheat, spring wheat and oat to *P. graminis* and other respective rust species (Mansfield et al., 1997).

In Fusarium wilt of chickpea, seven races have been reported throughout the world based on their differential reactions to separate host lines of chickpea. These races have been found to be geographically variable, such as Race 0 is reported in California, Spain and Tunisia, Race 1 is widespread in central India while Race 2 occurs in northern India (Phillips, 1988; Haware and Nene, 1982). On the other hand, in case of *Colletotrichum lindemuthianum* of the common bean, isolates belonging to common geographic isolates have shown genetic similarity. In fields, where one main type of cultivar is grown for several years, only single pathotypes are identified (Gonzalez et al., 1998).

Molecular markers are being increasingly used to characterize fungal plant pathogen populations in terms of genetic diversity and phylogenetic relationships within and between species. Additionally, markers that are closely linked to avirulence genes are being increasingly sought to assist map based gene cloning. Consequently, the development of DNA markers using RFLP, RAPDs, SSRs etc. has initiated mapping of genes contributing towards complex traits to be mapped using quantitative trait locus (QTL) analysis (Jones et al., 1995). The advanced DNA fingerprinting method of amplified fragment length polymorphism, AFLP (Vos et al., 1995) combines the power of RFLP analysis with the flexibility of PCR based technology. The technical advantages of AFLP over other fingerprinting methods are: (1) AFLP markers are neutral and detect variation over the entire genome (Majer et al., 1996), (2) The system is rapid and efficient (Vos et al., 1995) and (3) AFLP fingerprints are reproducible and reliable. Primer annealing is highly specific as primers are homologous to restriction site sequence and adapter sequence. Also high annealing temperatures at the initial cycles of the PCR ensure specificity as the primers differing in only one nucleotide or two nucleotides (e.g. E+A, E+AG) in the extension step produce distinct banding patterns (Mueller et al., 1996; Majer et al., 1996). Without prior knowledge of genomic sequences, AFLP can differentiate highly related strains in accordance to existing taxonomic data (Janssen et al., 1996). A few examples of the applications of AFLP among fungal pathogens are identification of mating type genes (Julian et al., 1999), determination of sex locus in fungi to unearth the mechanism of sex differentiation (Reamon-Büttner et al., 1998), clonality in fungi as means to reproduction (Rosendahl and Taylor, 1997) and discovery of new species involved in complex diseases (Baayen et al., 2000).

The downy mildew pathogen exists as populations in a field and hence prediction of damage or warnings against epidemics to farmers is highly complicated. Variation in the pathogen populations contributes to the understanding of pathogenesis and could answer queries of the pearl millet-downy mildew interaction. Our group has in the past attempted to screen pathogen populations with microsatellite and RAPD markers and to cluster them according to their fingerprinting profiles (Sastry et al., 1995; 2001). In my study, I used AFLP as a tool to detect variability among *S. graminicola* isolates from various geographical parts of India The objectives of my experiments were to (i) develop an efficient fingerprinting protocol for *S. graminicola* using AFLP, (ii) analyze the genetic variation among different isolates and, (iii) classify them into representative groups obtained by dendrogram and PCOA plot.

2.2 Material and Methods

Description, maintenance and growth of fungal isolates:

The isolates that I selected belong to different geographic regions of India (Table 3), and some were collected between 1997-1998 by Rao VP, ICRISAT.

Table 3 List of pathogenic isolates with given numbers, names and geographic origins.

Lane No.	Isolate	Host	Geographic origin
1	NHB3	Six host genotypes maintained on respective host genotypes by repeated inoculation with asexual spore in isolation chambers in green house also called Path 1-6 (Thakur et al., 1992).	
2	BJ104		
3	MBH110		
4	852B		
5	700651		
6	7042S		
7	*Sg* 32	HB3	Rahuri, Maharashtra
8	*Sg* 25	BK560	Bhadgaon, Maharashtra
9	*Sg* 26	Nath 4209	Veelad, Maharashtra
10	*Sg* 21	MLBH 104	Ghari, Maharashtra
11	*Sg* 48	7042S x HB3	Mysore, Karnataka
12	*Sg* 110	CO3	Illupanatham, Tamilnadu
13	*Sg* 139	Nokha local	Jodhpur, Rajasthan
14	*Sg*152	Lokal	Durgapur. Rajasthan
15	*Sg* 153	7042S x HB3 (AP)	ICRISAT Patancheru, Andhra Pradesh
16	*Sg* 88	GK 1006	Fatiabad, Maharashtra
17	*Sg* 140	Local	Jamnagar, Gujarat
18	PT2		
19	PT3	Mating type isolates	

I maintained the pearl millet downy mildew pathogen isolates from Indian states of Maharashtra, Rajasthan, Gujarat, Tamilnadu, Andhra Pradesh and Karnataka (Figure 6) on seedlings of their respective host cultivar or on a susceptible cultivar 7042 S in isolated chambers in greenhouses at ICRISAT, Patancheru. These isolates were multiplied through asexual generations. Other pathogen isolates, Path-1 to Path-6 were differential host

specific genotypes while pathogen isolates PT2 and PT3 were compatible heterothallic mating types (Michelmore et al., 1982).

Figure 6 Map of India illustrating *Sclerospora graminicola* (*Sg*) isolates collected from the represented states of India. Map not to scale.

I adopted the method of harvesting zoospores as described by Singh et al. (1993): Leaves were detached from infected 7042S plants and gently rubbed with cotton wool to remove old sporangiophores. The washed leaves were placed in plastic chambers lined with moist blotting paper and incubated at 20°C for 7 h. The incubators were programmed to lower the temperature thereafter automatically to 5°C. The mature sporangia were harvested in

tap water by gently brushing the leaf surfaces with a camel's hair brush. This inoculum was checked under a haemocytemeter and for further growth and maintenance of the pathotype, the sporangial suspension was made to a concentration of 1×10^6 mL^{-1}. Potted seedlings of respective host cultivar at coleoptiles–to-one-leaf-stage were inoculated by placing a drop of this freshly prepared, chilled (0-4°C, stored on ice) suspension at the tip of the seedling with a syringe. The room temperature for inoculation and overnight incubation was 20°C. Downy mildew chambers were used as relative humidity could be controlled to > 95% for increasing susceptibility of sporangial infection. The size of the pots was 6 inches; the pots were kept on greenhouse benches in isolation chambers at 25-30°C. The potting mixture used had components such as Alfisol, sand and farmyard manure in a ratio of 3:2:2 (w/v) with a small amount of diammonium phosphate (DAP) at 1 g kg^{-1} of soil. Seedlings were evaluated regularly for infection symptoms and to remove uninfected plants. Infected plants showing clear symptoms of chlorosis and located distant from each other were left in the pots to grow. Sporangia were harvested for DNA extraction after the plants were approximately 3-6 weeks old.

DNA extraction

I carried out the fungal DNA extraction according to the method described by Sastry et al. (1995). Sporangia were harvested from sporulating leaves in ice-cold sterile deionised water and a pellet collected by centrifugation. The pellet was powdered in liquid nitrogen and incubated with pre-warmed 5 volumes of extraction buffer (50 mM TRIS-HCl, pH 8.0, 20 mM EDTA, 0.5 M sodium chloride, 1 % sodium dodecyl sulphate) for 20 min at 65°C. A 1:1 volume of phenol-chloroform (chloroform : isoamyl alcohol were in 25:1 proportion) was added and gently mixed, followed by centrifugation for 10 min at 12000 g. The aqueous layer was further treated with equal volume of chloroform : isoamyl alcohol (also in 25:1 proportion) and centrifuged. The nucleic acids were precipitated from the aqueous phase by adding 0.6 volumes of isopropanol. DNA was spooled, washed in 70 % ethanol twice, dried and dissolved in $T_{50}E_{10}$ buffer (50 mM TRIS-HCl, 10 mM EDTA, pH 8.0). RNAse treatment and further purification was also followed. DNA dissolved in $T_{10}E_1$ buffer (10 mM TRIS-HCl, 1 mM EDTA, pH 8.0) was checked for concentration (O.D. 260/280) and quality.

AFLP analysis

The protocol that I employed for AFLP analysis was as reported by Vos et al. (1995). Research kit for AFLP of plant genomic DNA was from Life Technologies, USA and

assays were carried out as described in the manual. Basically, 150-200 ng genomic DNA was incubated with 1U *Eco*RI/*Mse*I mix for 90 min at 37°C with 1X Reaction Buffer. Digestion was followed by inactivation at 95°C for 2 min, the aliquot was distributed in two equal parts for ligation. Solution containing equimolar concentration of adaptors as given in the kit and T4 DNA ligase were mixed and incubated at 20°C for 2 h. The ligated sample was diluted 1:10 and 2 µl was used for pre amplification with 8µl pre-amplification mixture, 1 U Taq polymerase (Promega Corporation, USA) and 1X buffer. Selective amplification was carried out with 1:50 diluted preamplified mix, using [γ^{32}- P] labeled selectively modified *Eco* RI primer, selective *Mse*I primer containing dNTP mix, 1 U *Taq* polymerase, 1 X Buffer and AFLP grade water as described in the manual. Reaction cycles were carried out in Perkin Elmer 9600 Thermocycler. The amplified samples were mixed with 98 % formamide and xylene cyanol-bromophenol blue dyes, heated at 96°C for 5 min, ice cooled and loaded in continuation on 6 % polyacrylamide gel for pre run at 80 W according to the standard method described for DNA sequencing (Sambrook et al., 1989). After electrophoresis, amplification products were viewed by autoradiography and scored for polymorphism.

DNA sequences of *Eco* RI and *Mse*I primers were as per the kit specifications. Selective amplification was done with five *Eco* RI primers with two selective nucleotides e.g. E + AC, E + TG etc. and seven *Mse*I primers with three selective nucleotides e.g. M + CAG, M + CAA etc. I screened totally 14 combinations as described in Table 4 and just the clear and unambiguous polymorphic bands ranging between 130–400 bp were scored.

Table 4 ***Eco* RI and *Mse* I primer combinations (Life Technologies, USA) used in the Amplified Length polymorphism analysis.**

EcoRI Primers→	E + AC	E + AG	E + TG	E + TC	E + AA
MseI Primers↓	M + CAT	M + CTG	M + CAG	M + CAC	M+ CTC
	M + CAG	M + CTC	M + CTG	M + CTC	M+ CAA
	M + CTC	M + CAT			
	M + CTG				
	M + CTA				
	M + CAA				

I used pearl millet DNA from cultivars NHB3 and universal susceptible host 7042S as controls in the AFLP reactions to avoid artifactual polymorphism from the plant DNA as this is a sensitive technique and parts of leaf sections are often isolated along with sporangia. It was endeavored to select only clear and unambiguous polymorphic bands ranging between 130–400 bp for my study.

Data analysis

I manually scored each selected AFLP fragment as present (1) or absent (0) for each pathogen isolate. The calculation of genetic distances was done according to the DICE coefficient (Sokal and Michener, 1958) with the formula

$$GS = \frac{2N_{xy}}{N_x + N_y}$$

Where N_{xy} is the number of shared fragments between two pathogen isolates X and Y and N_x and N_y are the total number of fragments present in isolates X and Y, respectively.

Based on the GS (genetic similarity) values, I performed a cluster analysis using the UPGMA (unweighted pair group method of averages) procedure and the software package NTSYS pc 2.0 (Exeter Software, East Setauket, NY). The dendrogram was drawn and plotted with the help of consecutive commands *SimQual* with coefficient DICE, *SAHN* with clustering method UPGMA, and *TreePlot.*

I obtained a graphical representation of the estimated genetic similarities between pathogen isolates by principal coordinate analysis (PCOA) from NTSYS pc 2.0 based on the above calculated similarity matrix as described by Gower (1966). The consecutive commands *Dcenter* using the GS matrix as input, *Eigen*, and *2Dplot* were used to generate the two dimensional PCOA plot.

2.3 Results

Banding patterns of isolates using AFLP primer combinations

Reproducible bands were obtained by the analysis of 14 AFLP primer combinations and totally 184 bands were polymorphic with an average of 13 bands per primer combination.

The combinations E + AC / M + CAA, E + AA / M + CTC, E + TC / M + CAC and E + TG / M + CTG sets gave the most polymorphic banding patterns. One representative of AFLP fingerprint is depicted in Figure 7 using E + AC / M + CAG primer combination. As shown here, the patterns between the pathogenic isolates are polymorphic, revealing one set of scorable fragments no.1 to 6.

Marker numbers 43, 49 and 58 from the primer combination E + AC/ M + CAA; markers 69, 76 and 77 from the E + TC/ M + CAC set; marker 162 from the E + TG/ M + CTG set and markers 122, 130 and 134 from the E + AA/ M + CTC set exhibited notable polymorphism as they could divide the isolates in a similar fashion as the dendrogram. This limited set can be selected to determine the character/function related to host-pathogen interaction, the loci linked to pathogenicity and virulence. Furthermore, fragment numbers 9, 11, 12, 13, 19, 22, 40,41, 42, 43, 49, 58, 69, 76, 77, 95, 101, 105, 122, 130, 132, 134, 149, 152, 162, 175 and 182 could remarkably distinguish the isolates. These belong to different primer combinations and the fragment sizes range between 250-450 bp.

Clustering of the isolates

Based on the dendrogram obtained using the similarity matrix resolved by the DICE coefficient, the selected Indian isolates could be divided into two main clusters separated by a threshold genetic distance of 0.54 (Figure 8). The first cluster includes subclusters of the following isolates: subcluster of Path 5, *Sg* 25 and *Sg* 88; subcluster of *Sg* 110 and *Sg* 153; subcluster of Path 1; *Sg* 152 and *Sg* 139 and Path 3 distinctly. The second cluster includes the following isolates that form subclusters: sub cluster of Path 6, *Sg* 32 and *Sg* 26 and subcluster of *Sg* 21, *Sg* 48, *Sg* 140 and PT2. Path 2, PT3 and Path 4 form single isolate subclusters. Within the sub cluster, the isolates *Sg* 140 and PT2 show high genetic similarity i.e. GS = 0.84.

The PCOA plot in Figure 9 shows the various isolates form two distinct groups (I and II) with subgroups A and B in each (Figure 9). Group IA consists of mating type isolate PT2; west Indian isolate *Sg* 140; *Sg* 48 from Karnataka and *Sg* 21 from Ghari region of Maharashtra (similar to the subcluster in dendrogram analysis).

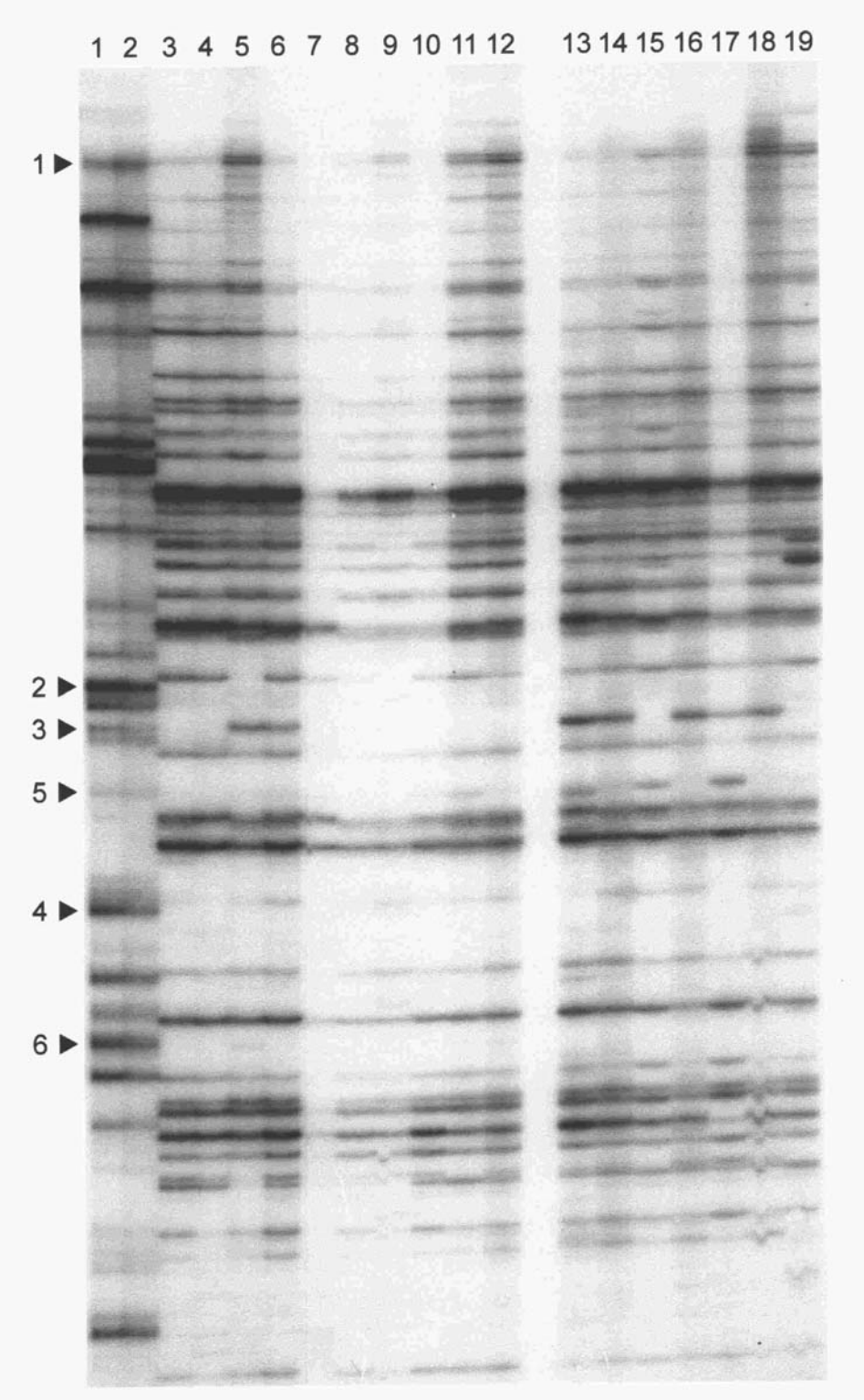

Figure 7 **A typical AFLP profile of the 19 isolates of *Sclerospora graminicola* (on horizontal axis) using the primer combination E + AC, M + CAG (Life Technologies, USA). The arrows point out to Markers 1 to 6. Refer to Table 3 for description of the isolates. Lanes 1 to 19 represent various isolates of *S. graminicola* as given serially in Table 3.**

Group IB includes the other known mating type isolate PT3, host genotype specific isolates Path 2; Path 4; Path 6; *Sg* 32 from south west India, Rahuri and *Sg* 26 from Veelad (refer to map in Figure 7). Group IIA involves isolates from geographically proximate regions in Rajasthan, *Sg* 152 and *Sg* 139. Group IIB comprises of host genotype specific isolates Path 3; Path 5; *Sg* 25 from south western isolate Bhadgaon, Maharashtra; *Sg* 153 from Patancheru (ICRISAT), Andhra Pradesh; *Sg* 110 southern isolate from Tamilnadu and *Sg* 88 from Fatiabad, Maharashtra. When I related the obtained figure to known information on the isolates, it was realized that in the PCOA plot, the first coefficient classified the host genotype specific isolates into two groups while the second coefficient separated the isolates into two mating types. Geographically distant populations were also separated; for example Rajasthan population of Group IIA was separated from the Maharashtra isolates spanning groups IA, IB and IIB. The genetic variation was in accordance with the cultivars from which the isolates were collected and from their characteristic virulence attributes.

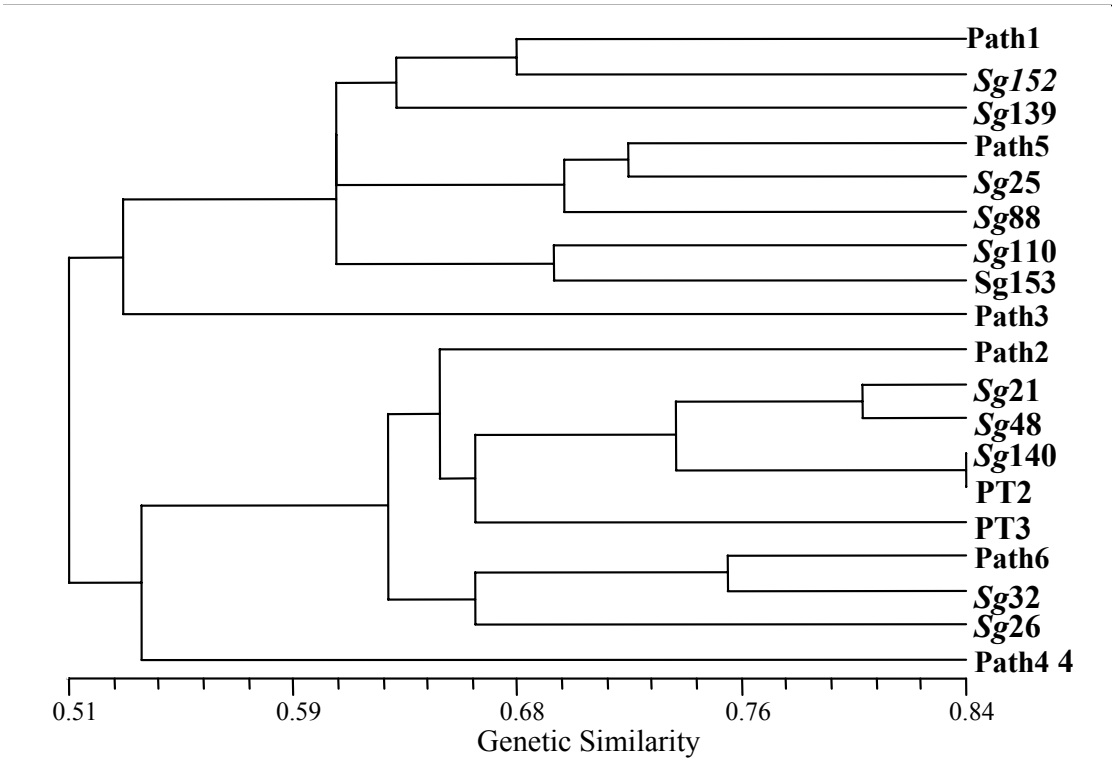

Figure 8 **Dendrogram based on AFLP polymorphisms in 19 pathogen isolates of *Sclerospora graminicola* of pearl millet by the unweighted pair group method of averages (UPGMA). Genetic similarities were calculated using DICE coefficient in the *SimQual* module of the NTSYS-pc 2.0, based on the 184 band positions observed for 14 AFLP primer pairs.**

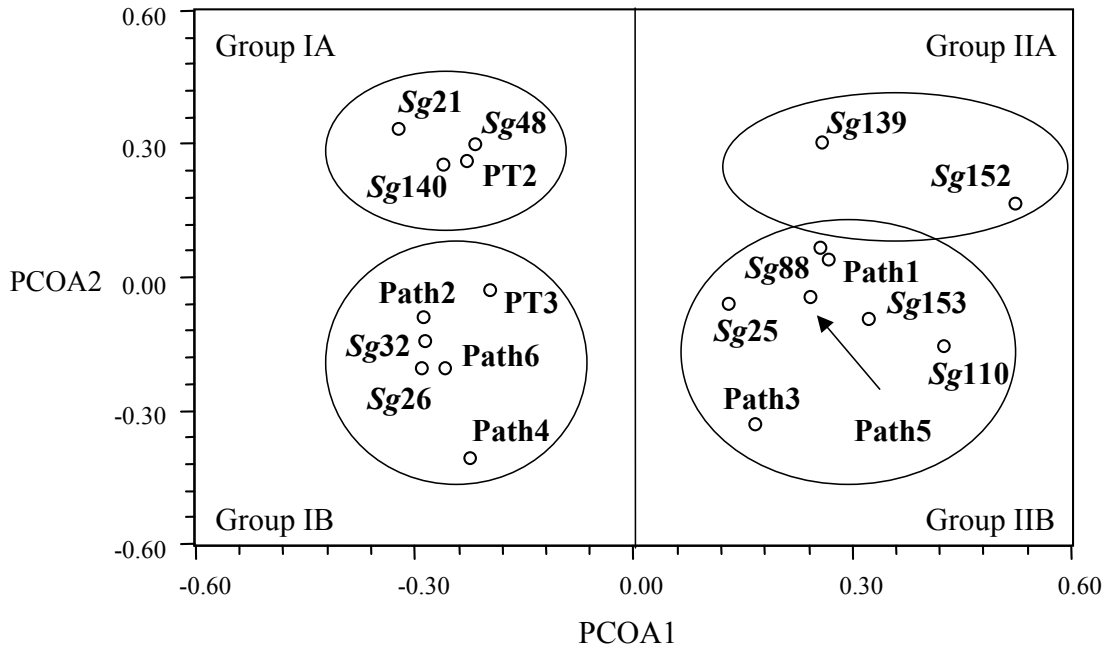

Figure 9 Differentiation of 19 downy mildew isolates by 2-dimensional Principal Coordinate analysis (PCOA) based on genetic similarity calculation from 14 AFLP primer combinations.

2.4 Discussion

The classical pathotyping based on biological traits indicates the occurrence of diversity in the fungal pathogenic populations of *S. graminicola* in aspects such as host specificity, adaptation and virulence (Thakur et al., 1992). When biological pathotyping is supported by genetic similarity, accurate information about the variation in aggressiveness of the isolates in relation to each other and to the host can be secured. Previous studies by my laboratory have explored adaptation of pathotypes to alternate hosts over asexual generations using RAPD approach (Sastry et al., 2001), identification of repeatable elements using restriction digestion (Sastry et al., 1997) and RFLP fingerprinting of the six host specific pathotypes (Sastry et al., 1995). In RFLP, many different restriction enzymes and combinations have to be used while RAPD assays are difficult to reproduce, since the PCR conditions for RAPD are of low stringency and thus prone to variations. The PCR conditions in AFLP analysis are high stringency based (Tyler et al., 1997; Vos et al., 1995) and AFLP method advantageously requires only one restriction enzyme combination and less amount of genomic DNA (150 ng) which can be competently organized with sporangia collected from field isolates over long distances. My study has provided a

successful method to find AFLP related markers for investigating the genetic diversity among *S. graminicola* isolates.

Heterothallism supported by AFLP analysis

Several published reports have presented many fine points of breeding for disease resistance in pearl millet and have emphasized the usefulness of information of genetic structure of the host and pathogen populations for durable resistance against downy mildew (Andrews et al., 1985; Hash et al., 1997 and Jeger et al., 1998). *S. graminicola* is a self-sterile, heterothallic organism, needing oogonium and antheridium for sexual fusion and the existence of two sexually compatible mating types, PT2 and PT3 is established (Michelmore et al., 1982; Ball and Pike, 1983). In my analysis using AFLP, these mating types fall in two distinct groups IA and IB as seen in the PCOA plot (Figure 9) and subclusters of the dendrogram (Figure 8). Heterogeneity in terms of virulence and aggressiveness has been previously reported among these isolates (Thakur and Shetty, 1993). Interestingly, it is yet to be known whether any other isolates have a similar genetic basis for mating type ability or virulence. Three isolates namely *Sg* 21 collected from south central part of India, *Sg* 48 from southwestern part of India and *Sg* 140 from western part of India cluster with the PT2 mating type isolate in Group IA. Among these, Idris and Ball (1984) have shown that *Sg*140 is sexually compatible and my AFLP data supports the view that it is not an isolated occurrence. It will be remarkable to observe if the virulence peculiarities of *Sg* 21 and *Sg* 48 isolates resemble PT2 (work in progress, Thakur et al., ICRISAT). The fine level subclustering observed among these isolates, however, may be due to their dissimilar host origins (Table 3): for example *Sg* 21 is isolated from MLBH 104, a popular high yielding pearl millet hybrid released in India; *Sg* 48 is from 7042S and HB3 hosts maintained in downy mildew nursery at Mysore and *Sg* 140 from a local variety.

Group IB (Figure 9) includes the second known mating type, PT3; host genotype specific isolate Path-2 (parent BJ 104, hybrid cultivated on a large scale for its agronomic traits, resulting in disease pressure, subsequent removal from cultivation); Path-4 (parent 852B); Path-6 (the most susceptible parent 7042S, a landrace originated from Africa); isolates from relatively adjoining geographic regions *Sg* 26 and *Sg* 32 (refer to map in Figure 7). The aggregation of these isolates in Group IB could probably be attributed to their analogous fertility to cross breed and magnitude for rapid virulence change (Thakur et al., 1998). Idris and Ball (1984) have been demonstrated that although populations differ in

pathogenicity, like Path -2, -4 and -6, such isolates can cross breed and cause rapid proliferation of pathogen genotypes in the absence of appropriate resistance factors in the host. The isolate, *Sg* 26 has been reported to have intra-population pathogenic variability like Path-3 and *Sg* 21 which were pandemic in the past leading to the withdrawal of the hybrids (Thakur et al., 1998). *Sg* 26 has also been found to be aggressive on the hosts of Path-4 and Path-6 of this group. Michelmore et al. (1982) and Idris and Ball (1984) have tried categorizing single oospore isolates according to the mating type compatibility and found homothallism frequent among these isolates. The relative frequencies of the mating types could be affected if oospores were inadvertently transported from one continent like Asia (India) to Africa presenting a risk to pearl millet crop. In summary, ascertaining of sexual compatibility of isolates will be of importance in determining the nature of durability of pearl millet to resist downy mildew pathogen.

Geographic relatedness among the isolates

Geographic relatedness is a frequent mode of classifying pathogen populations and this set could be termed as a gene pool (Stappen et al., 2000). In my analysis, group IIA of the PCOA plot (Figure 9) signifies the isolates from neighboring geographic areas, for example *Sg* 139 from Jodhpur is an aggressive isolate from a cultivar bred locally at Nokha and *Sg* 152 from NHB3 in a disease nursery at Durgapur. It is further reported that both have host specificity for pathogenicity (Singh and King, 1988). Group IIB comprises host genotype specific isolates Path-1; Path-3 and Path-5; *Sg* 25 from Bhadgaon, central India; *Sg* 88 from Fatiabad, central India; *Sg* 110 from south India and *Sg* 153, a field isolate from a disease nursery at ICRISAT, Patancheru. A similar observation has been made by Pei et al. (2000), where they have discovered that collection sites of rust isolates divide them into common groups based on cluster analysis and outbreak of new pathotypes from these populations. Furthermore, isolates *Sg* 32 and *Sg* 26 from Group IA (Figure 9) and *Sg* 25 and *Sg* 88 from Group IIB belong to nearby locations of southwestern and central India (refer to map in Figure 7). Closer physical distance accounts for the partial genetic homogeneity within the group, as oospores can spread rapidly (Williams, 1984). The genetic separation of these isolates probably suggests diversification of the pathogen coupled with the out-breeding nature of host giving rise to corresponding changes in pathogen genotypes.

Demonstration of high-level genetic variation in populations of *S. graminicola*

The AFLP data generated in my thesis has demonstrated a high level of genetic variation in natural populations of *S. graminicola.* From the breeding point of view, this could indicate rapid selection of isolates that are resistant to fungicides or virulent on pearl millet varieties having pathotype specific resistance. Selection of pyramided virulence has been manifested to be more efficient where the pathogen is an obligate biotroph, can reproduce sexually and the disease can spread rapidly by air borne sporangia (Day, 1974), traits which *S. graminicola* possesses. Separation of host genotypes specific pathotypes, Path-1, -2, -3, -4, -5 and -6 into two distinct clusters, IA and IIB, is indicative of the adaptable pathosystem to match the changes in host. These isolates are maintained on specific genotypes at ICRISAT (Thakur, personal communication) to find the virulence change from Path-6 to different isolates through several asexual generations in the greenhouse studies (Sastry et al., 2001). The host genotypes of Path-1 and Path-2 share a common male parent, HB3 (Mehta and Thakur, 1985) and both are isolated from high yielding varieties. However, Path-1 is much more aggressive than Path-2 (Thakur, 1995) and this could account for their grouping to different clusters. Path-3 and Path-4 show large differences in virulence ability, phenotypic changes over asexual generations (Singh and Thakur, 1995) and logically have large genetic distance between them as per our AFLP analysis. The virulence of Path-3 has been linked to Path-1, but not to Path-4 (Thakur et al., 1992), which is very well supported by my current data. Path-5 is isolated from a highly resistant host genotype, 700651, an accession that originates from Nigeria (Singh and Gopinath, 1985) in contrast to Path-6, the most aggressive downy mildew isolate known, is collected from a universal susceptible host. These two isolates belong to two different groups. Agronomic concern about Path-2 and -5 has grown as their hosts that have desirable crop qualities, and exhibit drought tolerance, are being implemented in a major program to incorporate downy mildew resistance in these pearl millet lines (Govila, 1993). Considering the causes of breakdowns of resistance from the epidemics of 1984-85 (Singh and King, 1988), Path-2 and -5 were checked for their virulence and aggressiveness and were found to have variable disease incidence (Thakur, 1995). In my work, I have shown that these isolates are genetically distant from each other, grouping in separate clusters of the PCOA plot.

Variation in *S. graminicola* isolates with similar host origins

In the dendrogram, I would like to point out that both Path-1 (also called NHB3) and *Sg* 152 have a common host, NHB3 (Thakur, 1995) (Table 3, Figure 9), and share genetic similarity. The combined influence of new cultivars, step-wise selection for virulence and mutable mechanisms of genetic re-assortment may have contributed to the development of new virulent races. Path-6 (also called 7042S) and *Sg* 153 (called field isolate 7042S) are ICRISAT, Patancheru populations and have a common host 7042S. Their high virulence capacity is used to test new pearl millet releases in breeding programs. Similarly, Path-2 and *Sg* 25 have the identical male sterile parent (5141A) and *Sg* 32 and *Sg* 48 have a common host (HB3) but group in different sub-clusters of the PCOA plot. The fact that these isolates have shown variation, supports that AFLP can detect differences at a very fine scale even in clonal populations. From the evidences of Ball et al. (1986) who found the inconsistent disease incidence of the two isolates and the present evidence that these are genetically dissimilar, one can explain why the isolates do not conform to the general pattern. The pattern refers to differences in disease incidence to establish consistency in pathogen behavior to fit host-pathogen cross relationships. In other host-pathogen systems also, namely *Magnaporthe griesea*-rice (Ziegler et al., 1995), perfect correlation between host and genotype is seldom observed (Gonzalez et al., 1998).

Advantages of using dendrogram and PCOA plot to represent diversity

In my analysis, the combined strategy of representations of dendrogram and PCOA plot has allowed calculation of genetic similarity to specific units and easy visualization of differences, respectively amidst collected sporangial populations to two main groups. Comparing the two methods of data presentation, the sub-clustering of dendrogram slightly differs from the PCOA plot. Both methods have used data generated from 184 amplicons wherein the PCOA plot revealed marked features of the isolates such as geographic relatedness, linking *Sg* 139 to *Sg* 152 in group IIA while separating mating types PT2 and PT3 to form individual groups. The dendrogram shows that Path-3 and -4 are notably different from each other as well as from the other isolates in the same groups and this has been pathotypically observed (Thakur et al., 1998). Sastry et al. (1995) have demonstrated that Path-3, -4; -5 and -6 fall in distinct groups with Path-2 clustering closer to Path-6 and that is well supported by my PCOA plot. They have also shown Path-1 and Path-2 to be genetically closer. On the whole, however, Path-1 and -2 fall in different groups as revealed by the dendrogram (Figure 8) and PCOA plot (Figure 9) of my analysis, which

demonstrates the importance of choosing correct primer pairs to reveal polymorphisms among pathotypes that are related. This also strengthens the use of different fingerprinting techniques like RFLP and AFLP to support each other.

In summary, my efforts on DNA fingerprinting by AFLP have facilitated the molecular description of the genetic differences between sporangial isolates thus providing a basis for further investigation of mechanisms that generate variation in fungi. Calculations of genetic distances have effectively identified divergent sub-populations from inter- and intra-regional sources that could harbor valuable genetic variations not apparent in current holdings of the isolates. In future, downy mildew has to be continuously monitored for the emergence of new races or pathotypes (Duncan and Milano, 1995) that can be fought with stable resistance genes of the host. Identification of markers associated with specific genes for host specificity, race specificity, virulence and avirulence will be the targets of research hereafter. Finally, refined AFLP markers would be useful for mapping and are speculative SCAR marker candidates for detecting polymorphism in this endeavor.

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Chapter 3

Identification of P5 retrotransposon like element in *Sclerospora graminicola* and contemplation of its role

3.1 Introduction

The fungus *Sclerospora graminicola*, like many other plant pathogens, displays considerable genetic diversity as reported by previous studies using RFLP, microsatellites and repetitive DNA (Sastry et al., 1995, 1997). The genetic variability can be owed to several factors such as sexual heterothallism (Michelmore et al., 1982), gradual changes over asexual generations (Sastry et al., 2001), evolutionary stress (McClintock, 1984), and presence of transposable elements (Finnegan, 1989). The genomes of all carefully studied fungal species are well known to contain transposable elements (TEs), which provide flexibility to populations to adapt successfully to environmental conditions (Kempken & Kuck, 1998). The movement of transposons constitutes to be a major source of spontaneous mutations and might be an important cause for genetic instability in fungi. Eukaryotic transposons can be divided into two major classes based on their mode of propagation (Finnegan, 1989). The class I type elements transpose by reverse transcription of an RNA intermediate and induce stable mutations which are called retrotransposons and are divided into two sub-groups, comprising elements with or without long terminal repeats (LTRs). In contrast, the class II elements lack the intermediate RNA and induce unstable mutations transposed via DNA-DNA mediated mechanism (Daboussi, 1997; Kempken & Kuck, 1998). Among these elements, retrotransposons are reported to contribute up to 80% of spontaneous mutations in fungal species (Dobinson et al., 1993; Anaya & Roncero, 1995).

Retrotransposons, resembling the integrated copies of retroviruses flanked by self-regulatory long terminal direct repeats (LTRs), contain an internal domain encoding *gag* and *pol* polyproteins (Toh et al., 1985). The *pol* protein has conserved domains characteristic of integrase, reverse transcriptase and RNAse H. Due to the functions of the domains of a retrotransposon, it can multiply or transpose independently using the genomic machinery of the host organism (Kumar et al., 1997). By sequence homology and order of these domains, retrotransposons are divided into two groups: Ty1-*copia* and Ty3-*gypsy*. Ty1-*copia* is the most studied group of LTR retrotransposons, named after the best characterized elements in *Saccharomyces cereviseae* (Ty1) and *Drosophila melanogaster* (copia). Table 5 illustrates retroelements such as Flipper, Grasshopper, Guest, Prt 1, EgR1, P5 etc. (Levis et al., 1997; Dobinson et al., 1993; Yeadon & Catcheside, 1995; Ruiz- Perez et al., 1996; Wei et al., 1996; Mc Hale et al., 1989) that have been strategically studied to estimate their presence in obligate oomycetes and their role in host–pathogen interactions.

Further, their contribution towards specificity to the host as well as adaptability to its equally changing host for further propagation of the pathogen has also been described (Gonzalez et al., 1998 and Kearney et al., 1988).

Table 5 List of some pathogenic transposable elements.

Transposable Element	Function	Source	Reference
1. Flipper	Transposase in nitrate reductase gene	*Botrytis cineria*	Levis et al. (1997)
2. IS801	Transposition of an avr ORF	*Pseudomonas syringae* pv. *Phaseolicola*	Gonzalez et al. (1998)
3. MAGGY	Gypsy-like LTR retrotransposon	*Magnaportha grisea*	Farman et al. (1996)
4. IS476	Transposon mutating avrBs1	*Xanthomonas campestris* pv.	Kearney et al. (1988)
5. Grasshopper	Long Terminal Repeat	*Magnaportha grisea*	Dobinson et al. (1993)
6. Guest	First reported non-transposon	*Neurospora crassa*	Yeadon and Catcheside (1995)
7. Prt 1	Retro transposon (RT) like sequence	*Phycomyces blakesleeanus*	Ruiz-Perez et al. (1996)
8. EgRI	No sequence similarity	*Erisyphe graminis*	Wei et al. (1996)
9. P5	Similarity to RT of *D.melanogaster*	*Fulvia fulva*	McHale et al. (1989)

Two strategies have been used to identify fungal retroelements. The first strategy involves cloning various repetitive sequences, and comparing to known transposons from other species although it is not known if these transposons are still active. Second is the transposon trapping approach, which is based on spontaneous inactivation of cloned genes so that active retrotransposons can be studied (Daboussi, 1997). Most systems studied to date have adopted *in vitro* mutation experiments facilitated by culturing the fungus in the laboratory. To date, there is only one example of a pathogenic retrotransposon mutating a plant's gene, thereby invading the host and securing survival (Kearney et al., 1988). In this case, the *Xanthomonas campestris* pathovar *vesicatoria* overcame a genetically defined

resistance in pepper, *Capsicum annuum*, by the transposon-induced mutation of *avrBs1,* a bacterial gene. These examples lead to a fundamental objective in studying host-pathogen interactions to determine how a pathogen can evolve to overcome the defense of a previously resistant host.

P5, a copia like element from *Fulvia fulva*, has been extensively reported in mammals, *Drosophila* and plants but less characterized in fungi (Grandbastien, 1998; Daboussi, 1997). In my study, I used this P5, a 225 bp element, having 30-100 related copies, some of which exhibit molecular polymorphism, to identify similar clones from downy mildew's λgt11 genomic library and to study its expression at the RNA level.

3.2 Material and Methods

Fungal and plant material

The isolates of *S. graminicola* were maintained on seedlings of pearl millet genotypes by repeated inoculation with asexual spores in isolation chambers in greenhouses at ICRISAT. Harvesting procedures were as described in Chapter 2, Materials and Methods. Five host genotype specific pathotypes, namely Path-1 (NHB3), Path-2 (BJ104), Path-3 (MBH110), Path-4 (852B) and Path-6 (7042S) (Thakur et al., 1992) were analyzed. The RNA studies were conducted using inoculation of Path 6 on susceptible pearl millet cultivar (cv) 7042S (exhibits about 90 % infection) and resistant cv 7042R (exhibits < 10 % infection). Inoculation and growth of seedlings were as described in Chapter 2, Materials and Methods section (Singh et al., 1993).

Isolation of Fungal DNA and RNA

Total fungal DNA was isolated as previously described in Chapter 2, Section 2.2, Materials and Methods section (Sastry et al., 1997).

Zoospores and 6 days old pearl millet seedlings were used for RNA isolation. At this stage, the former exhibiting chlorosis could distinguish the difference between susceptible and resistant seedlings. RNA was extracted by modifying the Chomczynski and Sacchi (1987) method. All glassware and plastic ware materials were rinsed with 0.1 % diethylpyrocarbonate and autoclaved at 15 p.s.i for 1 h to make them RNAse free. To 2 gm liquid nitrogen crushed, plant or sporangial tissue, a 10 ml Solution D containing 4 M guanidinium thiocynate, 25 mM trisodium citrate, 0.5 % sarkosyl and 0.1 M mercaptoethanol was added. 2 M sodium acetate pH 4.0, 10 ml phenol (equilibrated with water to pH 5.5-6.0) and 2 ml chloroform-isoamylalcohol mix were added next and the

mixture was gently mixed and incubated on ice for 15 min. A centrifugation at 10, 000 rpm or 3000 g was carried out at 4°C for 15 min to separate the DNA from RNA, the latter was collected in the supernatant and was treated with an equal volume of isopropanol and stored for 1 h in –20°C freezer. This was followed by a centrifugal run and the RNA pellet was collected to repeat the treatment with Solution D, sodium acetate and isopropanol. The pellet was washed in 80 % ethanol, dried and re-suspended in dH_2O for lithium chloride treatment. To this solution, 2 M lithium chloride was added and left overnight at 4°C. Pure RNA was collected by centrifugation at 10,000 rpm, for 20 min at 4°C. The pellet was re-washed with lithium chloride, centrifugation repeated and the leftover fine pellet was rinsed with 70 % ethanol and dried under vacuum. The quality and quantity of RNA were checked by electrophoresis and spectrophotometer. RNA obtained was treated further with lithium chloride and purified in ethanol. Treatment with RNAase I free DNAse I was done using GenHunter Corporation (Nashville, USA) enzyme and protocol. Ethidium bromide staining of rDNA bands was evaluated to ensure equal loading of RNA in each agarose gel lane. The agarose gel lanes contained 20 µg RNA isolated from the seedlings of infected susceptible and resistant plants, with 0 h and sporangial RNA as controls. RNA was transferred onto Nylon membrane (Hybond, N^+, Amersham Pharamacia Biotech) by capillarity using 0.4 N sodium hydroxide capillary buffer. The membranes were further used for hybridization experiments.

Northern analysis

Northern analysis was carried out using P5 as probe as described by Sambrook et al. (1989). The probe was purified by spin column method using grade II Ballotini balls (Jeneons) and sepharose CL 6B in the following manner: A hole was pierced in the bottom of a 0.5 ml eppendorf tube with a 25 gauge syringe needle. 50 µl of grade II Ballotini balls which had been previously washed and stored in Tris – EDTA (1M and 10M) respectively were topped with suspended sepharose CL 6B (Pharmacia) and the eppendorf tube was placed inside a 1.5 ml eppendorf tube. This apparatus was placed inside a 10 ml Falcon tube and centrifuged at 1000 g for 5 min to pack down the sepharose. 1ml of carrier DNA (autoclaved 5 gm salmon testes from Sigma, suspended in 1 lt distilled water) was placed in a 10 ml tube and the probe to be purified was loaded on the top of the assembly. This was centrifuged at 1000 g for 5 min and then the assembly was dismantled. When the Geiger reading was taken, the assembly was found to contain half as radioactively ‘hot’ as the falcon tube containing the isolated probe. The probe was used for radioactive random labeling.

The hybridization buffer was composed of 0.5 M disodium hydrogen phosphate (adjusted to pH 7.2 with hydrogen peroxide), 7 % sodium dodecyl sulphate and 1 mM EDTA and 100 μg/ml salmon sperm DNA (Sigma chemicals). Hybridization was carried out at 65°C for 12–16 h., however for stringent RNA hybridization, in presence of 50 % formamide the hybridization temperature was 42°C. Washes were given using 2× SSC (1 lt stock 20× SSC was composed of 175.3 gm sodium chloride and 88.2 gm sodium citrate adjusted to pH 7.0 with few drops of 10 N sodium hydroxide and brought to the required volume) twice at 60°C and once at room temperature for 10 min each to remove background non-specific hybridization. The membranes were exposed to X-ray films (Kodak X 100) for overnight or stored in –80°C freezer and the autoradiographs were later developed.

Plant DNA extraction

The pearl millet leaf DNA was isolated according to the procedure of Sharp et al. (1988). 2 gm of tissue was incubated in extraction buffer (5 μM Tris, 0.1 M EDTA, 5 μM sodium chloride and 1 % sodium dodecyle sulphate) for 1-2 h at 55°C. An equal volume of phenol was added, mixed and centrifuged at 5000 g. The supernatant was processed further with phenol – chloroform – isoamyl alcohol (25:1 v/v ratio of chloroform to isoamyl alcohol and equal volume of this mixture with phenol) and centrifuged. To the supernatant, an equal volume of isopropanol was added and the DNA was spooled. The dried DNA was dissolved in high salt buffer (50 mM Tris – 1 mM EDTA) and was given the RNAse treatment. It was further purified with phenol-chloroform and precipitated in isopropanol. The DNA concentration was determined on gel and with spectrophotometer, and the DNA was stored at –80°C freezer.

Genomic library construction

λ gt 11 genomic library of *S.graminicola* was constructed in our laboratory by Sastry et al. (1997) using Gigapack II packaging extract (Stratagene, La Jolla, USA). The DNA from sporangia of highly virulent isolate, 7042S (Path 6) was isolated as described in Chapter 2, Section 2.2 and was digested with *Eco*RI to obtain fragment sizes less than 4 kb. The genomic DNA fragments were further purified and ligated to λ gt 11 arms according to the manufacturer's protocol. Effectiveness of the ligation reaction was checked by electrophoresis on 0.6% agarose gel. Recombinant lambda phage arms were packaged using packaging extract and then adsorbed to bacterial strain Y1090R. The cells were plated to check packaging efficiency, which was found to be 1×10^7 recombinant plaques ml^{-1}. The adsorbed bacterial cells were plated on 150-mm NZY (Hi Media, India) agar

media plates with NZY top agarose. The plates were incubated at 37°C overnight and stored at 4°C for 2-3 h. For screening the library, the plaques were transferred to nitrocellulose filters and colony hybridization was carried out. The plaques of interest were isolated and suspended in SM buffer (0-1 M sodium chloride, 0.0105 M magnesium sulphate, 50 mM TRIS / HCl, pH 7.5, 0.01 % gelatin) with drops of chloroform and screened further for purification. The library was screened for retrotransposons using P5, a 225 bp (Mc Hale et al., 1989) cloned sequence. Five rounds of screening were undertaken to obtain five dissimilar clones. All the experimental details were according to the instructions from Stratagene manual and Sambrook et al. (1989).

Plaque purification of bacteriophage

The plating of bacteria was carried out by placing 10 ml sterile rich NZYM medium (NZ amine 1 %, 0.5 % sodium chloride, yeast extract 0.5 %, magnesium sulphate 0.2 % adjusted to pH 7.0 with sodium hydroxide) supplemented with 0.2 % maltose in a sterile tube and inoculating with Y 1090 R *E. coli* bacterial colony. The culture was grown overnight at 37°C with agitation at 250 cycles/min for efficient adsorption of the bacteriophage. The cells were centrifuged at 4000 g for 10 min at room temperature. The supernatant was discarded and the pellet re-suspended by vortexing with sterile 0.01 M magnesium sulphate. The suspension was used up within 3 weeks, though plating efficiency of freshly prepared cells was found to be better. For plating the bacteriophage, the following procedure was carried out: a 10 × serial dilution of bacteriophage stocks was made in SM buffer; 0.1 ml of each dilution was dispensed into sterile tube; 200 µl of the plating bacteria prepared as described were added to each tube. The bacterial phages were allowed to adsorb onto bacteria by incubating for 20 min at 37°C. Molten agarose (42°C), at the concentration of 0.7 %, was added to the first tube, gently vortexed and immediately poured on a labeled plate containing 30-35 ml hardened bottom NZY agar. The plates were let to stand for 5 min at room temperature to allow the agarose to harden. Plaques that appeared after 12-16 h were used for plaque screening with nylon membranes or for DNA preparation.

Phage DNA isolation

For the purpose of DNA isolation, the phages were allowed to adsorb overnight onto *E. coli* cells. 1 % chloroform was added to enhance lysis and transferred to a beaker without chloroform and most of the debris was removed by passing through muslin cloth. DNAse (Sigma Chemicals) was added to a concentration of 10 µg/ml, mixed and

incubated for 45 min at 37°C. Then sodium chloride powder was added to a final concentration of 1 M (5.84 g for 100 ml lysate). The lysate was passed through a four-fold muslin cloth and centrifuged at 3000 g at 6°C. The lysate was decanted to a plastic beaker and polyethylene glycol (8000) was added to a final concentration of 7 %. The mixture was incubated for 60 min and then centrifuged at 3000 g for 12 min at 6°C. The phage pellet was obtained and the supernatant was discarded. The pellet was dissolved in 800 µl 10 mM TRIS – 1 mM EDTA and transferred to eppendorf tubes. For each 400 µl lysate, 3 µl sodium dodecyl sulphate (20 %), 6µl 0.5 M EDTA, 30 µl Pronase and 10 µl RNAse were added, mixed and incubated for 30 min at 37°C, followed by 60°C incubation for 10 min Phenolysis was carried out by adding phenol and keeping eppendorf tubes on ice to precipitate polyethylene glycol. This was followed by chloroform separation. The phage DNA was obtained as a precipitate and was further purified using 2 volumes absolute ethanol and sodium acetate, followed by 70 % ethanol washes. The DNA pellet was dried and dissolved in 10 mM Tris – 1 mM EDTA. The concentration of DNA was measured on 0.8 % agarose gel and using spectrophotometer. From the DNA, the inserts were isolated by PCR, using forward and reverse primers of the phage. The details of a PCR run on 9600 Perkin Elmer were: a 94°C initial denaturation, followed by 35 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min followed by a final extension at 72°C for 5 min. The amplified fraction was run on 1.5 % agarose gel and the inserts were purified by Qiagen insert purification kit and also by the salt purification protocol. The inserts were used for cloning into plasmid vector by electro-shock (Life Science equipment).

Cloning of the genomic library fragments

Effectively, I obtained five clones from the genomic library screening that hybridized with the P5 clone (kindly gifted by Richard P. Oliver) and named them as *Sg*P5-1, *Sg*P5-2, *Sg*P5-3, *Sg*P5-4, *Sg*P5-5. The clones were isolated by PCR amplification with λgt11 forward and reverse primers, sub-cloned in pMOS blue (Amersham, Life Science, England) and were checked for blue and white colonies after transformation by electro-shock (Life Science equipment). The white colonies were selected on antibiotic ampicillin – Luria broth (1 lt of the media contained, 10 gm tryptone, 10 gm sodium chloride, 5 gm yeast extract w/v) and plasmids were isolated as described in the following paragraph.

Plasmid isolation by the alkali lysis method

The bacterial cells grown in 50 ml Super broth (1 lt contained 32 gm tryptone, 20 g yeast extract, 5 g sodium chloride, the solution dissolved in distilled water and adjusted to

pH 7.4) were harvested by centrifugation at 4000 g for 10 min at 4°C. The pellet was re-suspended in 10 ml solution I (50 mM glucose, 25 mM Tris, pH 8.0 and 10 mM EDTA, pH 8.0). A 20 ml of freshly prepared solution II (0.2 N sodium hydroxide and 1 % sodium dodecyl sulphate) was added and was gently mixed, followed by storing for 5 min. After this, 15 ml of Solution III (5 M potassium acetate mixed in 11.5 ml glacial acetic acid and 28.5 ml distilled water) was added, mixed and stored for 5 min before centrifugation at 4000 g for 20 min at 4°C. Six volumes of isopropanol was added to the supernatant and the mixture was allowed to stand at room temperature for 15 min. The DNA was recovered in the pellet by centrifugation for 15-20 min at 5000 g at room temperature. The pellet was washed with 70 % ethanol and dried under vacuum. RNAse (10 μg / ml) treatment was given after the pellet was dissolved in TE. The plasmid was further purified by sodium chloride (1.6 M) – polyethylene glycol 8000 (13%) treatment, precipitated by aliquots of 10 M ammonium acetate and 100 % ethanol followed by washes with 70% ethanol and dried. The pure plasmid pellet was used for quality and quantity estimation on 1% agarose gel and spectrophotometer, where optical density, 1 O.D = 50 μg.

Insert purification

The inserts that I obtained from the bacteriophage were purified by the salt extraction method from agarose gel. DE 81 paper (Whatman) was used to ionically load the inserts directly from the gel by electrophoresis. The paper was checked under ultraviolet light to ensure complete transfer of the insert on to it and then taken out and packed into an eppendorf column that was pre-packed with glass wool into an eppendorf with a small hole pierced by a paper pin at the bottom of the tube. 100 μl of 1.5 M sodium chloride, pre-warmed at 55°C, was added to the column and the apparatus was incubated at 55°C for 20 min. With a brief spin, the DNA solution was centrifuged down into another eppendorf tube. Again 100 μl of 1.5 M sodium chloride was added and the same steps repeated 3 times to get totally 4 fractions of the purified insert. 2 volumes of absolute ethanol were used for precipitation at –70°C. After a centrifuge spin, the pellet was washed twice in 70 % ethanol, dried and dissolved in 10 mM TRIS – 1 mM EDTA and used for labeling or running on gel for electrophoresis.

The inserts that I obtained from the plasmids were isolated by QIAquick gel extraction protocol using a microcentrifuge (Qiagen, Germany, instruction protocol). The insert DNA after amplification from bacteriophage was run on agarose gel and excised with a clean, sharp scalpel. The gel slice was weighed in a colorless tube and 3 volumes of buffer QX1

were added to 1 volume of gel (to 100 mg gel slice 100 µl were added). This was incubated at 50°C for 10 min until the gel slice completely dissolved. The color was checked and was found to be yellow. To this, 1 gel volume isopropanol was added and mixed. A QIAquick spin column was placed in the provided 2 ml collection tube. To bind the DNA, the sample was applied to the QIAquick column and centrifuged for 1 min. The flow-through was discarded and the QIAquick column was placed back with same collection tube. 0.5 ml of the buffer QX1 was added to QIAquick column and centrifuged for 1 min. To wash, 0.75 ml of buffer PE was added to QIAquick column for an additional 1 min at 10,000 g. The QIAquick column was placed into a clean 1.5 ml microfuge tube. To elute the DNA, 50 µl of 10 mM Tris-HCL, pH 8.5 was added to the center of the QIAquick column and allowed to stand for a few minutes. This was followed by centrifugation to recover the purified insert.

Restriction enzyme digestion and southern hybridisation

The *Hind* III digest of 10 µg fungal DNA was separated by electrophoresis through 0.8 % agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and visualized by ethidium bromide and ultra violet light. The gels were destained in water and transferred to nylon filters by Southern blotting. The filters were hybridized with respective DNA probes labeled with (α-^{32}P)– dATP (Sambrook et al., 1989). Random prime labeling kit (BRIT, New Bombay, India) was used for labeling the probes using the provided klenow polymerase, buffer and nucleotide mix. Hybridization signal was detected by an overnight exposure to X-ray film (Indu, India). The fixer solution used for developing the X-ray films composed of 200 gm sodium thiosulphate and a pinch of sodium metabisulphite dissolved in 1 lt water. The developer contained 2 gm Metol, 90 gm sodium sulphite (anhydrous), 8 gm hydroquinone, 45 gm sodium carbonate and 5 gm potassium bromide in a 1 lt volume made up by water. The mixing of the ingredients was done at 50°C in a comparative semi-darkness.

3.3 Results and Discussion

It has been known that retroelements contribute to almost 5-10 % of euakaryotic genome (Bingham & Zachar, 1989) and hence generate significant genetic diversity in plants and fungi by mutating the host organism's genes. They have also been shown to be factors responsible for genetic instability, playing an important role in dynamics of eukaryotic genomes (Kaneko, 2000). McClintock (1984) postulated that genomic stress could activate retroelements, based on the hypothesis that transposition could increase genetic variability

that is necessary for organisms to adapt to different stress conditions (Daboussi, 1996; Kempken & Kuck, 1998). Since then, several examples have proved this hypothesis correct in abiotic stress (Anaya and Roncero, 1996). Moreau-Mhiri et al., (1996) have also shown their activation by biotic microbial factors with common ability to elicit hypersensitive plant defense response.

A data base search has revealed that the archaic Ty1-*copia* retrotransposons-like sequences are closely associated with the euchromatic regions of chromosomes, where the active genes are mainly located (Wessler, 1995). On the other hand, many of the presently known retroelement-like sequences appear to be non-functional due to stop codons and frame-shifts introduced by mutations or by virtue of their localization in intergenic regions of the genome (SanMiguel et al., 1996). Such non-functional retrotransposons do not mutate genes at high frequency and avoid accumulation of lethal mutations minimizing the selection pressure upon them. The P5 retroelement has been first reported in *Fulvia fulva* as a *copia*-like retroelement and the mechanism of its mobility, as in case of other filamentous fungi, remains unproven. My investigation was initiated with an aim to comprehend the distribution of retrotransposon-like sequences among downy mildew isolates and their role in plant-pathogen interaction, specifically in pearl millet-downy mildew system.

Presence and distribution of P5-like sequences in *S. graminicola* genome

I have used a 225 bp amplicon of the P5 element to screen the phage genomic library of *S. graminicola*. The efficiency of the titer was 1×10^7 recombinant plaques ml^{-1}. From 30 plaques that hybridized with the P5 element in the first round of screening, I finally obtained four true positive plaques that were sub-cloned into plasmid vector. The sizes of clones named as *Sg*P5-1, *Sg*P5-2, *Sg*P5-3, *Sg*P5-4 were between 500 bp–1 kb. The inserts of these clones, amplified in PCR using λgt11 forward and reverse primers upon hybridization with P5 as probe, confirmed their selection but revealed bands with different intensities indicating their partial sequence homology to P5 element. In order to study the cross homology of the sequences, the insert from each of the four clones was hybridized with all the four inserts amplified by PCR. Figure 10 depicts a representative hybridization pattern of *Sg*P5-1 sequence with all the four inserts. The non-inclusion of any part of lambda vector DNA within the clones was checked using a λ DNA control (Lane 1, Figure 10), which did not show hybridization with *Sg*P5-1 fragment. The differential

hybridization patterns obtained demonstrated the partial homology of the P5 sequence among these four sequences.

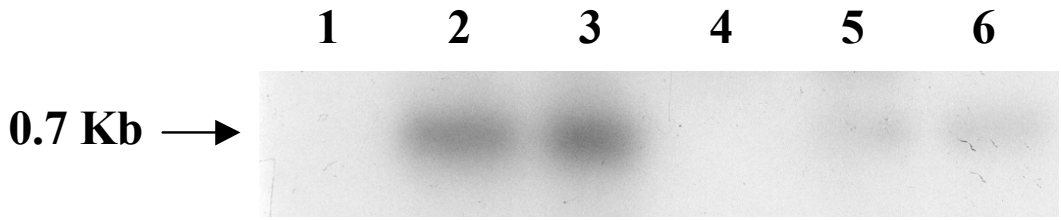

Figure 10 Hybridization of phage clones *Sg*P5-1, -2, -3, -4, (lanes 2, 3 and 5, 6) with PCR amplified insert of genomic library clone *Sg*P5-1. Lane 1 represents uncut λ DNA and lane 4 is blank.

The distribution of P5-like sequences was analyzed among five host genotype specific downy mildew pathotypes. Figures 11 A and B represent the hybridization patterns of *Sg*P5-2 and *Sg*P5-4 inserts with *Hind* III restriction enzyme digested DNA of the five pathogenic isolates. The probes detected mostly single hybridizing bands in all the five pathotypes however; the band sizes were different with each probe. For example, *Sg*P5-2 showed a band of size 4.3 kb (Figure 11 A), while *Sg*P5-4 indicated a band of 6.0 kb in size (Figure 11 B). The hybridization intensity was different among the different isolates although the amounts of DNA loaded in each lane were same as confirmed by the hybridization of the same blots with rDNA probe. Thus, the differences in the hybridization signal intensity among these isolates were probably due to sequence divergence of the P5-related elements.

On the whole, a monomorphic pattern of the clones of P5 element-like sequences among the host genotype specific isolates is in agreement with the observation made by Mc Hale et al., (1989) wherein monomorphic patterns have been obtained in *Fulvia fulva* isolates when hybridized with P5 element. Similar results are also obtained by Kusaba & Tsuge (1994) who studied *Alternaria alternata*, the causal agent of black spot disease in certain cultivars of Japanese pear. The markers they obtained using RFLP of nuclear rDNA and nucleotide sequence variation in the internal transcribed spacer regions of rDNA (Adachi et al., 1996) could not differentiate different pathotypes. Although the sample size of my study is not sufficiently large to allow a definite conclusion, it is likely that P5 is distributed in all the downy mildew host genotype specific isolates. Furthermore, P5 element-like sequences cannot be used for diversity studies of host genotype specific *S.*

graminicola isolates due to the monomorphic patterns as against previous studies in my group using RFLP (Sastry et al., 1995) and AFLP (Singru et al., in press) where one could differentiate the host genotype specific isolates.

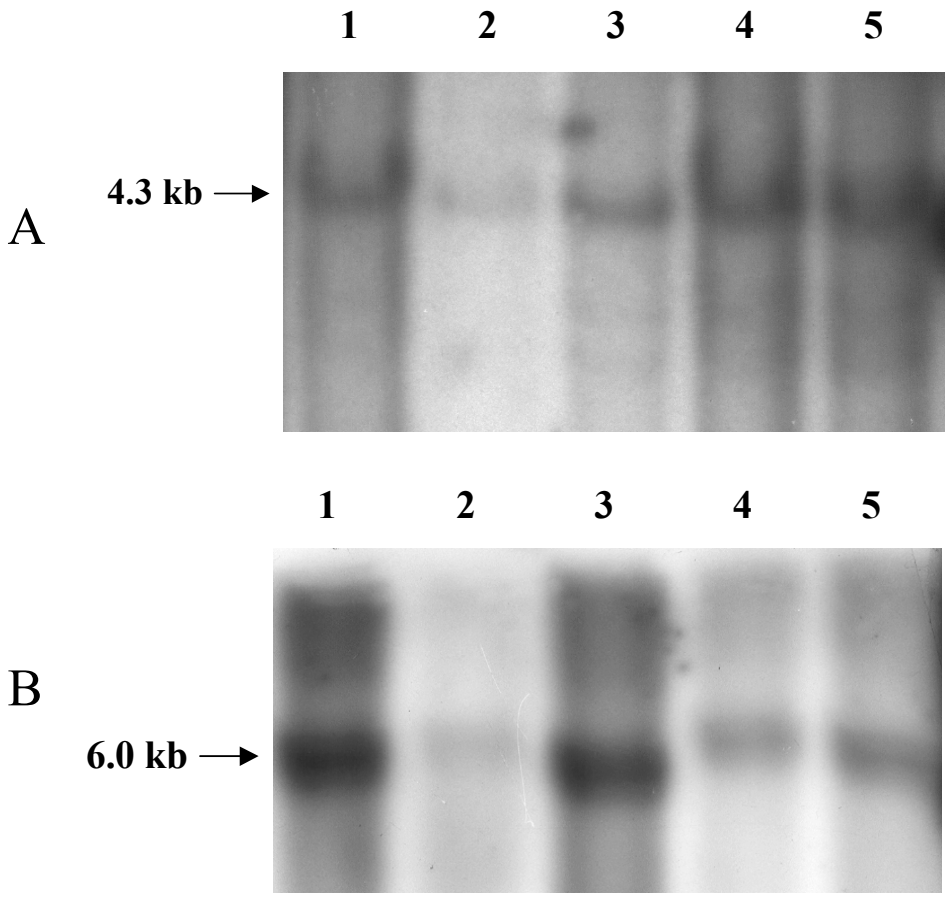

Figure 11 Southern hybridization using P5-like sequences from the genomic library as probes with *Hind III* restricted DNA from *Sclerospora graminicola* Pathotypes -2 , -3, -4, -5, -6 (lanes 1-5 respectively). A: hybridization using radio labeled probe *Sg*P5-2. B: hybridization using radio labeled probe *Sg*P5-4.

The distribution of retrotransposons has been extensively studied in *Magnaportha grisea* and *Botrytis cinerea* (Dobinson et al., 1993; Farman et al., 1996; Shull & Hammer, 1996). Three retrotransposons, MAGGY, *fosbury* and *grasshopper*, from *M. grisea* have appeared to be distributed in a host–specific manner. It has been inferred that these elements are horizontally transferred to the host genome concurrently with, or subsequent to, the evolution of each host – specific form of the retroelement. On the other hand, Boty LTR retroelement from *B. cinerea* appears to be not host specific as it is present in strains of *B.*

cinerea isolated from grapes and tomatoes. Similarly, Flipper retroelement from the same species is found in other phytopathogenic fungi like *M. grisea* and *Fusarium oxysporum* (Levis et al., 1997). In my study, the P5 retrotransposon originated from *Fulvia fulva* exhibits homology with another oomycete *S. graminicola*, thus indicating non-specificity of the host for this retroelement.

Does the P5 retrotransposon-like element play a role in pearl millet – downy mildew interaction?

Of the several retro elements known, only a limited number are transcriptionally active which probably limits the hazardous effects of growing populations of retrotransposons in the host genome (Casacuberta et al., 1997). I was keen to study if the P5 element-like sequence in *S. graminicola* was trancriptionally active and if so, whether it had any role in host-pathogen interaction. Retrotransposons evolve differently, particularly with respect to their expression features, if maintained under different conditions. Therefore, I conducted an experiment to study their active behavior in a natural system by maintaining the fungus on its host in greenhouse under natural conditions. Figures 12 A and B give a representation of the results obtained in my experiments.

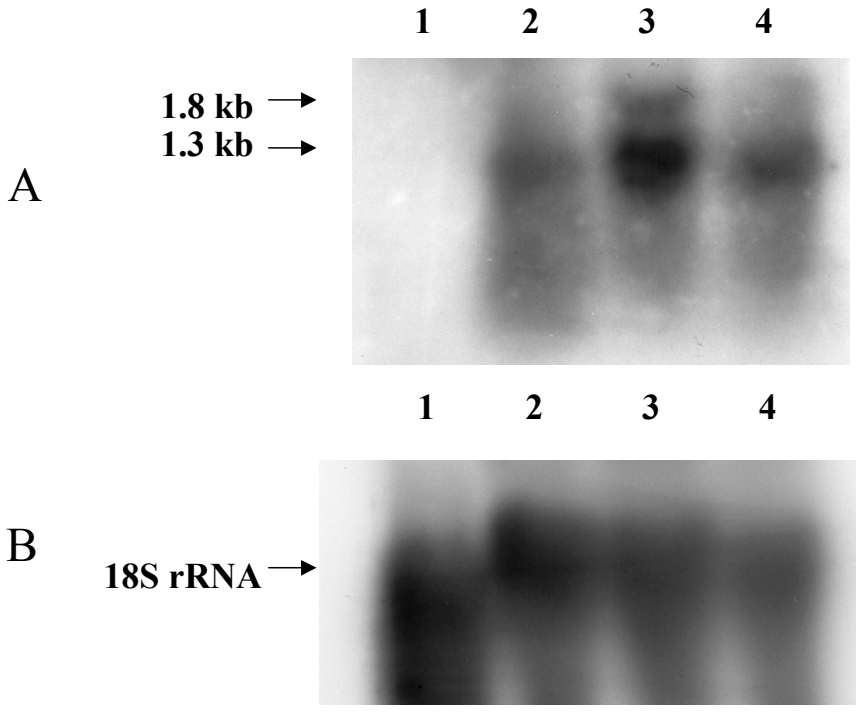

Figure 12 Northern hybridization with RNA from healthy and infected pearl millet cultivars. The RNA was collected from infected plants 6 d after

inoculation. Lane 1 represents 18S ribosomal DNA, lane 2 - healthy 7042S and 7042R, lane 3 - infected 7042S and lane 4 - infected RNA 7042R. A: hybridization using radio-labeled probe 18S rDNA. B hybridization using P5 radio-labeled probe.

At the RNA level, a band of 1.3 kb was observed in healthy control as well as in infected resistant (7042R) and susceptible (7042S) pearl millet on hybridization with P5, indicating a homology between the fungal and plant P5-like retrotransposon elements. Furthermore, resistant pearl millet did not display the high intensity signal, while susceptible variety showed an amplified expression as well as an additional band of size 1.8 kb. Re-hybridization of rDNA probe with the northern blot showing equal intensity bands (Figure 12 B) inferred that differences in hybridization signal between the infected resistant and susceptible pearl millet RNAs (Figure 12 A) were due to preferential hybridization of the P5 element to susceptible RNA and not due to variation in amounts of RNA loaded.

All genomes are endowed with systems for defense against intrusive DNA. Resistant plants exhibit a defense response against the pathogen through a network of stress-induced proteins, some of which inactivate fungal retro elements at DNA or RNA level (Grandbastien, 1998). In obligate parasitic fungi like *S. graminicola*, the fungus could express itself in the host as illustrated by the amplified level of P5-like sequences and a high molecular weight band of size 1.8 kb in susceptible cultivar 7042S (Figure 12 A). Biotic stress activation might allow elements to move in rare situations, without major effects on the organism's viability, a prerequisite for their own survival.

It is possible that on infection, the plant's intrinsic P5-like retroelement is amplified, which could have a role in susceptibility and which is suppressed in resistant cultivars. As the fungus cannot be grown axenically, it is difficult to quantify the expression differences between plant and pathogen during the process of infection. Thus, although it remains to be proved beyond doubt if P5 expression is linked to pearl millet's defense response, my experiments could potentially be considered as the first direct evidence for the involvement of retrotransposons in pearl millet – downy mildew interaction.

Summing up, retro elements are a major source of genetic variation that ranges from gross chromosomal alterations up to very fine tuning of the expression of cellular genes (Kunze et al., 1997) and are involved in the organisms' adaptability to environmental changes (Wessler, 1996). From my study, it can be postulated that P5-like sequences are present in the genome of *S. graminicola* that are putatively expressed at the RNA level in compatible

interaction of downy mildew with the 7042S cultivar of pearl millet. Further studies on characterization of active fungal retro elements will be necessary to understand their impact on disease and on plant genomes they attack.

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Chapter 4

Differential expression of plant defense genes in pearl millet - downy mildew interaction

Part A: Comparison of healthy and infected pearl millet tissues using DDRT approach

4.1 Introduction

Plants confronted with phytopathogens react to the challenge in a number of different ways. The infection of plant tissues by a pathogen initiates a complex progression of morphological, biochemical, molecular and physiological interactions that culminate in the visual symptoms that we associate with disease or disease resistance. The nature, timing and spatial coordination of the actions taken by either plant or pathogen are crucial in defining the result of any given interaction and thus the overall outcome of the infection. There are three kinds of host-pathogen interactions, namely, (i) non – host interaction which is responsible for the basic resistance in all plants, (ii) compatible interaction occurring between a susceptible host and its threatening pathogen resulting in disease and, (iii) incompatible interaction that takes place between a resistant host and its pathogen that cannot cause disease. A range of genes and intricate pathways that are unique to crop types control the disease mechanisms.

In plants, genetic control of resistance mechanism ranges from the action of single genes to the action of many genes simultaneously, whereas in the pathogen, pathogenicity and aggressiveness are inherited independently and are controlled by single and many genes. It is the interaction of all these genes in both host and pathogen along with environmental effects that generates the resistance profile of the plant. Strategies that might provide transgenic crops with a potentially more durable resistance towards a broader spectrum of pathogens are currently being investigated. These strategies involve pathogen-induced cell death and are often based on general defense responses occurring in plants during incompatible plant-pathogen interactions. For example, in potato the barnase-barstar two-component system could control cell death and thereby bring about the growth arrest of *Phytophthora infestans* (Strittmatter et al., 1995). Another two-component system explored the existing natural defense mechanisms in plants, the gene-for-gene model. In plants harboring a *R*-gene and the corresponding *Avr* under the control of a strictly pathogen-inducible promoter, resistance reactions are activated upon pathogen attack. Honee and coworkers (1995) have shown how the constitutive expression of the gene encoding *Avr*9 elicitor from *Cladosporium fulvum* in tomato plants carrying the corresponding resistance gene (*Cf*9) resulted in delayed growth, necrosis and even complete cell death.

Over the past years, a number of *R*-genes have been isolated which may be used to engineer resistance in susceptible cultivars. Wu and coworkers (1995) have transferred a gene encoding glucose oxidase from *Aspergillus niger* into potato plants which caused transgenic potato plants to produce higher levels of hydrogen peroxide, thus activating the production of phytoalexins and cross-linking of the cell wall proteins which have a role in hypersensitive response and thereby resistance. Mittler et al. (1995) have observed accumulation of defense-related transcripts, phenylammonia lyase (PAL), and other compounds and changes in ion fluxes in their studies on transgenic tobacco plants which, showed resistance towards tobacco mosaic virus and *Pseudomonas syringae*. Another strategy involves developing transgenic plants that express plant polygalacturonase-inhibiting proteins (PGIPs), which inhibit fungal endo-galacturonidases, thereby creating nonspecific elicitor molecules that activate defense responses (Toubart et al., 1992).

The most prevalent models that explain disease resistance do not always take into account the data obtained from specific plant - fungus interactions involving intact plants, suggesting that these models may be too simplistic, or not applicable in certain plant-fungus interactions. Pearl millet-downy mildew is a complex relationship where the downy mildew is a biotrophic parasite that cannot be cultured axenically in the laboratory and research has been limited as both host and pathogen are outbreeding in nature and highly variable. So far, some histological reports of progress of the disease in compatible reactions have made the identification and prediction of the disease possible (Sharada et al., 1995; Subramanya et al., 1983; Safeeulla, 1976).

Faced with the above challenges, I undertook two approaches namely the DDRT, differential display reverse transcriptase described in Part A of this chapter, and candidate genes described in Part B, to study the genes involved in pathogenesis. Other common methods for identifying differentially accumulated mRNAs in particular plant tissues at specific time intervals have been differential screening and subtractive hybridization (Cook et al., 1995). However, these methods require lot of biological material and are labor intensive. Differential display technique circumvents these problems (Liang and Pardee, 1992) and is also more sensitive (Zimmermann and Schultz, 1994). With this method, patterns of amplified cDNA products of different mRNA samples can be compared side by side on a denaturing polyacrylamide gel and thus differentially expressed cDNAs can be identified. These experiments require small amount of biological material, which is suitable for studying gene expression during early stages of infection when the leaves of pearl millet are in coleoptile stage.

In this part of my work, I have described how DDRT was used to compare healthy pearl millet samples to infected susceptible and resistant tissues at 8 and 72 h after infection and sporangial RNA. Since altered gene expression is expected in the plant and fungus from the onset of fungal invasion, the differential pattern were compared and the bands cloned in plasmid vectors for further studies.

4.2 Material und Methods

The following diagram schematically describes the application of DDRT as a method in perceiving pearl millet-downy mildew interaction.

Total RNA extraction and quantitation of healthy and infected pearl millet seedlings and sporangia

↓

cDNA preparation

↓

Radioactive PCR

↓

PAGE

↓

Excise differential bands

↓

Re-amplify

↓

Reverse Northern analysis

↓

Clone true positives in pGEM T easy vector

↓

Northern hybridization with the clones as probes

↓

Sequence the differential clones

↓

Functional genomics to find homology with known clones or establish novelty of the sequences

Plant and fungal materials

Pearl millet seed samples of resistant line (7042R, < 10% disease incidence) and susceptible line (7042S > 90% disease incidence) were grown in greenhouse as described in detail in Chapter 2, Section 2.2, Materials and Methods. Spores from Path 5, the isolate from Patancheru, were used for inoculation of 7042S seedlings. Inoculation of leaves with spores of downy mildew pathogen was done as described in Chapter 2, Section 2.2 (Singh et al., 1993). Seedlings are most susceptible to downy mildew infection, and hence the coleoptile to 72 h period was used for my analysis. Seedling samples were collected at 0 (healthy control), 8 and 72 h. Spores were included as control as the fungus is systemic and displays constitutive levels of related genes.

RNA extraction

RNA was extracted by modifying the Chomczynski and Sacchi (1987) method as described earlier in the Chapter 3, Section 3.2, Materials and Methods.

Differential display assay

Differential display analysis was carried out using RNAmap^{TM} (Genhunter Corporation, Nashville, USA). To synthesize cDNA, 2 µg DNA free RNA was incubated at 65°C for 5 min in a 20 µl reaction containing 5× RT buffer, 1 mM dNTP, 1 µM T_{12}MN (anchored oligo dT primer) followed by incubation at 37°C for 60 min, 95°C reaction termination for 5 min and cooled to 4°C until further use. PCR was carried out using a core mix of 2.0 µl from the earlier reaction, 10× PCR buffer, 0.1 mM dNTPs mix, random primers (Operon Technologies) and T_{12}MN primers according to instruction protocol, α ^{32}P-dATP and 10 U Ampli-Taq (Perkin Elmer Corporation). The PCR cycle was run on Perkin Elmer 9600 thermocycler at the following program: 94°C for 30 sec, followed by 2 min at 40°C, 30 sec at 72°C, the cycle repeated 40 times, followed by an extension at 72°C for 5 min and cooled to 4°C until further use. Several combinations of anchored and random primers were analyzed. Aliquots of the post PCR mixtures were heated at 94°C for 2 min in an equal volume of loading dye (95 % formamide, 0.2 % xylene cyanol and 0.2 % bromophenol blue) and separated on a 6 % polyacrylamide/8M Urea gel in 1X TBE buffer for about 3.5 h at 60 watts constant power until the xylene cyanol dye reached the bottom. The gel was transferred on 3M papers, covered with plastic wrap and exposed to X-ray film (Kodak) at - 80°C for overnight. The Operon primers namely K1, K2, K3, K4, K5,

K6, K7, K8, C1, C2, C3, C4, C5, C6, C19, C20, E4 in various combinations with anchored primers from GenHunter namely, $T_{12}MA$, $T_{12}MG$, $T_{12}MC$, $T_{12}MT$ were attempted.

Reverse Northern analysis of display products

Bands of interest were cut out of the dried gel along with the paper using a sterile scalpel, incubated in 100 µl of dH_2O for 10 min and eluted by boiling for 15 min. The supernatant was treated with 0.03 µM sodium acetate, 0.05 mg/ml glycogen and 450 µl 100 % ethanol, on dry ice for 30 min. After centrifugation, the pellet was treated with 85 % ethanol, briefly centrifuged and the residual ethanol was removed. The pellet was dissolved in 10 µl dH_2O and 40 % was used for re-amplification. The cDNAs were re-amplified in the same PCR conditions and with the previously used set of primers, without isotope. 20 µl of the PCR products were run on 1.5 % agarose gel, and the bands were purified using Qiagen (Germany) purification kit to separate the PCR product from primers and other non-incorporated nucleotides. Dot blots were prepared from 10 µl of the purified product and the DNA was transferred to Nylon membranes (Hybond N^+, Amersham Pharmacia Biotech) by capillarity (Sambrook et al., 1989) in 0.4 N sodium hydroxide. One membrane was hybridized with first strand cDNA from infected pearl millet 7042S and second membrane was hybridized with first strand cDNA from healthy pearl millet 7042R and 7042S (0 h samples).

Cloning and sequencing of cDNA fragments

The cDNAs were cloned into pGEM T easy vector (Promega) by electro-shock method (Life Sciences). Plasmids were prepared using Sodium dodecyle sulphate – alkaline lysis method as described in Chapter 3, Section 3.2, Materials and Methods (Sambrook et al., 1989). The clones were subsequently subjected to sequencing with sequenase enzyme (Amersham Pharmacia Biotech) using an automated system (AFLexpress, Amersham Pharmacia Biotech) with florescent labeled T7, SP6 primers. DNA sequences were edited to discard the vector/linker and primer sequences. Further treatment of the sequences could not take place due to unexpected changes in the vector/primer linkages.

Colony transfer

The nylon membrane (Hybond N^+, Amersham Pharmacia Biotech) was cut according to grid size and numbered with the clone number to be transferred. The bacterial colony containing the clone was inoculated as a dot with a toothpick. The subsequent colonies were transferred fast to prevent drying of the colonies. Once all the grids were inoculated,

the membrane was gently lifted and placed on LB media agar plate with antibiotic ampicillin. The colonies were allowed to grow overnight. The membrane was then removed and placed for 5 min. on Buffer Solution I such that it was floating and not submerged in the solution (Sambrook et al., 1989). Next, the membrane was placed in Solution II kept in a plastic box with a tight lid. It was 'cooked' at 85°C water bath for 6 min. After this, the membrane was placed on top of Solution III (0.5 M TRIS HCl, 0.5 M EDTA, 5 M sodium chloride, 20 % Sarcosine and 5 mg Proteinase K powder) and was incubated for 30 min at 37°C. The solution was decanted and the membrane was cross-linked under ultraviolet light and stored at 4°C until hybridization.

Northern analysis:

The cDNA fragments cloned in pGEM T easy plasmid vector (Promega) were used as probes. Inserts were excised from the vector by digestion with Eco RI, followed by electrophoresis on 1.5 % agarose gel in 1X TAE buffer. The inserts were purified from the gel using Qiagen insert extraction kit. The nylon membranes from northern analysis were hybridized using selected cDNA probes and radio labeled using Random Prime labeling kit (BARC, Jonaki, Secunderabad, India) with α-^{32}P-dATP, containing the provided nucleotide mix, random labeling buffer, klenow enzyme and radioactive label.

4.3 Results and Discussion

In my study, differential display was used to analyze gene expression during the process of infection by downy mildew in susceptible pearl millet. For this purpose, the RNA was extracted from 8 h and 72 h infected seedlings, along with healthy tissue and sporangial RNA. Of the many primer combinations tried to compare gene expression between the infected and healthy seedling, 15 primer combinations were short-listed. To reduce the number of false positives picked, a double Reverse transcriptase enzyme was used, the second being MMLV reverse transcriptase (Clontech laboratories). 70 of the selected cDNA products were re-amplified with original set of primers used for differential display as described in Materials and Methods. These re-amplified products were examined on 1.5 % agarose gel and some of the results are shown in Figure 13. Among these, 56 fragments were successfully re-amplified as distinct bands with no contaminating background and corresponded to the expected size, ranging from 400 to 600 bp.

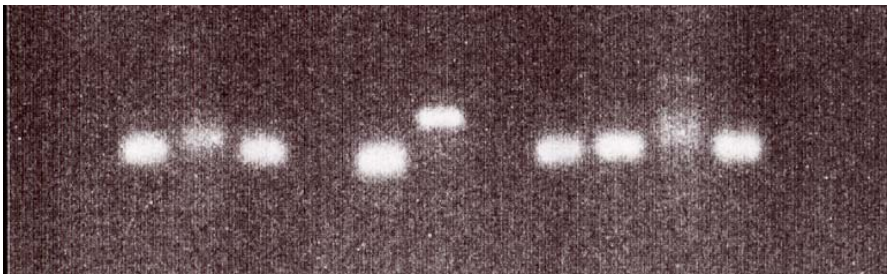


Figure 13 Gel electrophoresis of cDNAs amplified from DDRT – PCR. Lanes 1, 2 and 3 – T_{12}MA – K7, lanes 4 and 5 – T_{12}MG – C4, lanes 6, 7, 8 and 9 – T_{12}MC – K3. T_{12}MA, T_{12}MG and T_{12}MC are anchored primers (GenHunter) and K7, C4 and K3 are random primers (Operon).

Figures 14 A-D show the differential display patterns obtained from the sample using four different primer combinations namely TMA-K7, TMG-C4, TMC-C4, TMC-K3. The molecular sizes of the bands ranged between 400-600 bp. It is obvious from the arrows marked that the combination of primers had a significant effect on the banding pattern observed. Using the same RNA populations, the bands could be reproduced in two separate reactions symbolizing the true positives (differentially expressed cDNAs). The primer combination, TMC-K3 also revealed infection specific defense-related cDNAs present only in the 72 h sample (Figure 14 A). Primer combination TMA-KT revealed presence of bands in the healthy samples that were absent in the inoculated seedlings (Figure 14 B). When TMG-C4 primer combination was used, bands of about 500 and 450 bp were present only in 7042S infected after 8 h sample but absent in healthy and sporangial lanes, and absent in the 72 h sample (Figure 14 C). Primer combination TMC-C4 revealed pathogenesis specific bands at 8 and 72 h, which were absent in the controls (Figure 14 D). Some of the bands appear to be pathogenesis specific and phase specific, as they are expressed only in the infected samples. The results demonstrate the ability of the differential display method to detect in vitro differences in RNA populations between healthy, infected and sporangial tissues.

Reverse Northern analysis of differential display products

In differential display experiments, it is necessary to confirm that the genes corresponding to the screened fragments are truly differentially expressed. This is because it has been observed that differential display reactions generate a high percentage of false positives (Corton and Gustaffson, 1997). In my experiment, Reverse Northern analysis on dot blots

was used to identify clones that were specific to the infected samples. Several putative house keeping genes or fragments were eliminated that were common between healthy and infected samples.

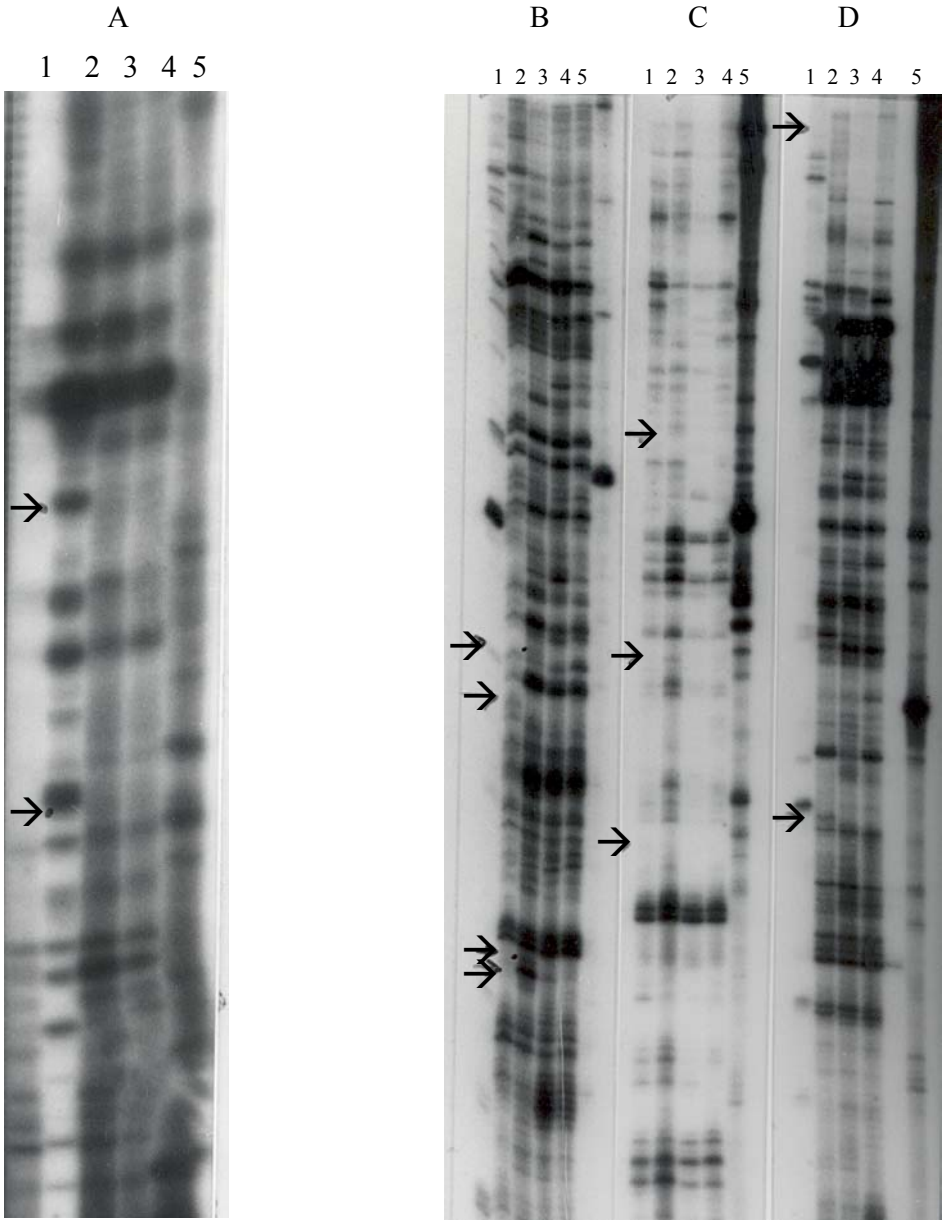


Figure 14 **A: DDRT profile using T_{12}MC-K3 primer combination. Lane 1 - sporangial cDNA, lane 2 - 7042S pearl millet cDNA collected 72 h after inoculation, lane 3 – 7042S pearl millet cDNA collected 8 h after inoculation, lane 4 – healthy control 7042R pearl millet cDNA and lane 5 – healthy control 7042S cDNA.**
B: DDRT profile using T_{12}MA-K7 primer combination. Lane 1 – 7042S cDNA collected 72 h after inoculation, lane 2 – 7042S cDNA collected 8 h after inoculation, lane 3 – healthy control 7042R cDNA, lane 4 – healthy control 7042S cDNA and lane 5 – sporangial cDNA. Please ignore lane 6.
C: DDRT profile using T_{12}MG-C4 primer combination. Lane 1 – 7042S

cDNA collected 72 h after inoculation, lane 2 – 7042S cDNA collected 8 h after inoculation, lane 3 – healthy control 7042R cDNA, lane 4 – healthy control 7042S cDNA and lane 5 – sporangial cDNA.
D: DDRT profile using T_{12}MC-C4 primer combination. Lane 1 – 7042S cDNA collected 72 h after inoculation, lane 2 – 7042S cDNA collected 8 h after inoculation, lane 3 – healthy control 7042R cDNA, lane 4 – healthy control 7042S cDNA, followed by a blank lane and lane 5 – sporangial cDNA.

Northern blot analysis

To further verify the authenticity of the differentially displayed bands, cDNAs clones corresponding to some of the selected bands were used to probe dot blots containing RNA from 7042S pearl millet tissue infected after 72 h and 8 h; healthy 7042S and 7042R; and sporangia. Some of these clones hybridized strongly with the 72 h and 8 h samples, and those that hybridized with healthy RNA were eliminated. Differential hybridization between 8 h and 72 h samples was also observed. Some showed mere intensity differences, which can account for phase specific as well as copy number variation. These cDNAs may represent pathogenesis related genes whose proteins are specifically associated with the process of disease formation in compatible reactions.

These clones would, therefore, be useful for further understanding of the process of infection in pearl millet. Some of these clones did not show hybridization in northern blots, which is possibly due to the fact that the original band excised from the gel, was contaminated by other co-migrating cDNA fragments of the same or similar molecular weight. As a result, the cDNA clone used as a probe for Northern analysis did not represent the RNA of interest. In this case, it would be necessary to select several individual clones to determine which of the clone gives the desired differential signal on Northern blots. Sequencing of the clones was tried but without success and it was contemplated that the clones were degraded or not fully subcloned and due to this technical shortcoming, sequence comparison to known homologous genes could not be done.

Differential display method has been used to identify novel genes enhanced by *Azorhizobium caulinodans* infection of its legume host *Sesbania rostrata*, from stem and root tissues after various time intervals of nodulation (Goormachtig et al., 1995). Intensity differences were also found in the clones at different time intervals. Partial sequences were observed that were highly homologous to hydroxyproline rich cell wall proteins, which shows the wide efficiency of DDRT technique. Thus it has been proven that one does not have to isolate the full-length cDNA to elucidate the role of gene expression and the

pathways that are novel to the plant. It is possible to propose the functions of the gene products that are enhanced and describe whether they are specific to the species, whether they are spatially specific and what inductive factors are responsible for their over expression. Marked cellular changes were observed during feeding site formation of the soybean cyst nematode (Hermsmeier et al., 1998), which were supported by abundant mRNAs differential display, though ultimate sequences realized that only a few were not house-keeping genes. These researchers found putative aldolase genes, transcription factor homologue, GTP-binding protein and an auxin down-regulated gene to have roles in the compatible interaction between soybean and its cyst nematode pathogen. Yet another example discriminating housekeeping genes from defense-related genes used mutants strains that had separated the fungal mycelia from the plant tissue, allowing the discovery of cutinase genes involved in plant-pathogen interaction (Munoz and Bailey, 1998).

Another advantage of the DDRT - PCR technique is that it permits the simultaneous identification of up and down regulated genes and hence it has been used to isolate mRNA species in plants induced in response to biotic and abiotic stress. The Table 6 shows some of the examples of the applications of DDRT – PCR.

Table 6 Cloning of genes using DDRT in host-pathogen systems: some examples.

Pathogen	Host	Gene(s)	Source
Botrytis cinerea, *Phytophthora infestans* and tobacco necrosis virus	Tomato	DDR, DD5 and DD47 with no sequence homology to any known sequence	Benito et al. (1996)
Colletotrichum trifolii	Alfa alfa	SRG1, SRG2-plant defense proteins and tree pollen allergens	Truesdell and Dickmann (1997)
Erisyphe graminis and *Rhynchosporium secalis*	Barley	HvGRP2-cysteine rich domain and HvGRP3-RNA binding domain	Molina et al. (1997)
Heterodera glycines	Soybean	Putative aldolase genes, transcription factor homologue and GTP-binding protein	Hermsmeier et al. (1998)

The usefulness of the method has been demonstrated in systems like *Botrytis cineria* - tomato (Benito et al., 1996) in which three new cDNA clones of the fungus were found to

be enhanced which were not produced in response to another pathogen namely, *Phytophthora infestans*. In *Phytophthora capsici* – pepper system (Munoz and Bailey, 1998) it was found that fungal cutinases were induced while the soybean cyst nematode-soybean interaction (Hermsmeier et al., 1998) uncovered down regulated genes that were organ specific. DDRT – PCR was also used to identify applications to general molecular pathogenesis in various fungal organisms (Sturtevant, 2000). Some flowering studies carried out at various flowering stages (Singh and Cheah, 2000) revealed eight flower specific cDNAs to differentiate from vegetative tissue of flowering palm. Fruit ripening of strawberry associated with non-climatic factors (Wilkinson et al., 1995) revealed developmental specific cDNAs and studies on nodulation (Goormachtig et al., 1995) identified differential infection specific root primordial cDNAs. An advancement of the DDRT – PCR was achieved by improving the rapidity and reliability from minute amounts of tissue from Loblolly pine embryos for the mass cloning and characterization of differentially expressed genes (Xu et al., 1997). In consequence, DDRT appears to be a useful tool for screening reactions in plants and my study could show some differential activity between healthy and infected pearl millet seedlings.

Part B: Candidate gene approach to study the genes involved in pathogenesis

4.4 Introduction

Genes involved in the plant-pathogen interaction are known as 'defense related genes' that are regulated constitutively or by parasitism (Heath, 1991). The signaling events that build up the inducible defense responses are: (i) local gene activation, that highlights a rapidly evoked induction of PAL (Phenylalanine ammonia lyase) (EC 4.3.1.5), lipoxygenase (EC 1.13.11.12), and a set of other defense molecules, and (ii) systemic gene activation which is triggered gradually and induces chitinase (EC 3.2.1.14) and various linked proteins. Defense-related genes code for proteins that directly or indirectly, by virtue of their catalytic activities, restrict growth and development of the invading pathogen.

PAL is a key enzyme involved in the general phenyl-propanoid pathway and regulation of phenolic biosynthesis. Potential compounds that are synthesized through PAL include flower and fruit pigments; UV-B filtering components; phytoalexins and cell wall components viz. wound lignins. All these are distributed essentially in all higher plants, some acting as defense agents by increasing the cell wall resistance to penetrating hyphae, hydrolytic enzymes and toxins of infecting fungi (Hahlbrock and Scheel, 1989). On induction by pathogen attack, PAL is often synthesized in specialized cells at particular stages of infection (Wada et al., 1995), its concentration changes remarkably giving an insight into the plant-pathogen interaction and the interplay of biochemical pathways (Cui et al., 1996).

Lipoxygenase catalyzes the oxidation of free fatty acids, and being denoted as a wound hormone, its role in seed germination and disease resistance has been cited (Galliard and Chan, 1980). Its peroxide like products have anti-microbial activity and volatile products are similar to fungicides (Siedow, 1991).

Chitinase has been clearly demonstrated to have anti-fungal activity (Schroeder et al, 1992) by hydrolyzing the fungal structural cell wall components after the pathogen penetrates the xylem vessels (Boller, 1987). The magnitude and speed of its induction appear to be important determinants of resistance.

In case of pearl millet, qualitative and quantitative resistance ranging from immunity to susceptibility has been observed towards downy mildew (Thakur, 1992). Deeper knowledge about defense-related genes could lead to a better understanding of the mechanisms involved in durable resistance and may define targets for improving disease

resistance. I, therefore, undertook studies on a few genes namely PAL, chitinase and lipoxygenase in pearl millet – downy mildew system at RNA level and tried to correlate temporal patterns of early events of *S. graminicola* infection on pearl millet seedlings based on these defense-related genes.

4.5 Material and Methods

Host and fungal material

Pearl millet seed samples of resistant line (cv 7042R, < 10 % disease incidence) and susceptible line (cv 7042S, > 90 % disease incidence) were grown in greenhouse according to the procedure described in Chapter 2, Section 2.2, Materials and Methods (Singh et al., 1993). Spores from Path 5, the isolate from Patancheru, were used for inoculation of leaves as described by Sastry et al., 1995. The most susceptible stage of downy mildew infection is the seedling stage, hence the coleoptiles to 6 d (144 h) period was used for analysis. Seedling samples were collected at 0 (healthy control), 4, 24 and 72 h except in case of At PAL where 8, 12, 48 and 6 d (144 h) samples were also included. Spores were included as control as the fungus is systemic and displays constitutive levels of related enzymes.

RNA extraction and gel blot analysis

RNA was extracted by modifying the Chomczynski and Sacchi (1987) method as described in the Chapter 3, Section 3.2, Materials and Methods section. Treatment with RNAase I free DNAse I was done using GenHunter Corporation (Nashville, USA) enzyme and protocol. Ethidium bromide staining of rDNA bands was evaluated to ensure equal loading of RNA in each gel lane. Each lane contained 20 µg RNA isolated from the seedlings at 0, 4, 24, 72 h after infection of susceptible and resistant plants, with 0 h and sporangial RNA being controls. In case of PAL, 8, 12, 48 h samples were also included. RNA was transferred onto Nylon membrane (Hybond, N^+, Amersham Pharamacia Biotech) by capillarity where the capillary buffer was 0.4 N sodium hydroxide.

Hybridization procedure

Hybridization probes used were PAL (a 6 kb gene clone from *Arabidopsis thaliana*, *At PAL*, gifted by C Lamb, Salk Institute for Biological Sciences, San Diego, USA), lipoxygenase (a 3 kb gene probe from tomato, gifted by AK Handa, Purdue University, West Lafayette, USA) and chitinase (a 1 kb gene probe from maize, CH11, gifted by JM Widholm, University of Illinois, Urbana-Champagne Urbana, USA). The sequences included in these probes are highly conserved across species boundaries and hence likely

to hybridize to the pearl millet genome. The probes were purified by spin column method using grade II Ballotini balls (Jeneons) and sepharose CL 6B in the following manner: A hole was pierced in bottom of a 0.5 ml eppendorf tube with a 25 gauge syringe needle. 50 µl of grade II Ballotini balls that had been previously washed and stored in Tris – EDTA (1M and 10M respectively) was added. This was topped with suspended sepharose CL 6B (Pharmacia) and the eppendorf tube was placed inside a 1.5 ml eppendorf tube. This apparatus was placed inside a 10 ml Falcon tube and centrifuged at 1000 g for 5 min to pack down the sepharose. 1ml of carrier DNA (denatured 5 gm salmon testes from Sigma, suspended in 1 lt distilled water) was placed in a 10 ml tube and the probe to be purified was loaded on the top of the assembly. This was centrifuged at 1000 g for 5 min and then the assembly was dismantled. When the Geiger reading was taken, the assembly was found to contain half as radioactively 'hot' as the falcon tube containing the isolated probe. The probe was used for labeling.

Hybridization buffer was composed of 0.5 M disodium hydrogen phosphate (adjusted to pH 7.2 with hydrogen peroxide), 7 % sodium dodecyl sulphate and 1 mM EDTA and 100 µg/ml salmon sperm DNA (Sigma chemicals). Hybridization was carried out at 65°C for 12 – 16 h, however for stringent RNA hybridization, in presence of 50 % formamide the hybridization temperature was 42°C (Sambrook et al., 1989). Washes were given using 2× SSC (1 lt stock 20× SSC was composed of 175.3 gm sodium chloride and 88.2 gm sodium citrate adjusted to pH 7.0 with few drops of 10 N sodium hydroxide and brought to a required volume), twice at 60°C and once at room temperature for 10 min each to remove background non-specific hybridization. The membranes were exposed to X-ray films (Kodak X 100) for overnight or longer, stored in –80°C freezer and the autoradiographs later developed. Quantification of hybridizing bands was performed using TINA 2.08 (Raytest Isotopemessgeraete GmbH). The hardware was based on IBM-PC and software was Windows XP. Background hybridization was measured by sampling autoradiograph area outside the lanes, and was subtracted from the signal of the hybridizing bands with the software. Data of signal intensities (measured as optical density, O.D) was calculated per mm^2. The actual density of the test sample (O.D minus background signal density per mm^2) was considered and the graphs were portrayed using ORIGIN 6.0 (Microcal Software Inc.).

4.6 Results

The resistant and susceptible pearl millet cultivars 7042R and 7042S were selected to determine the expression level and timing of transcript accumulation of three defense response genes encoding PAL, chitinase and lipoxygenase upon infection. The healthy control (0 h tissue) displayed no disease symptoms.

Transcript accumulation of defense-related genes in *S. graminicola* infected seedlings

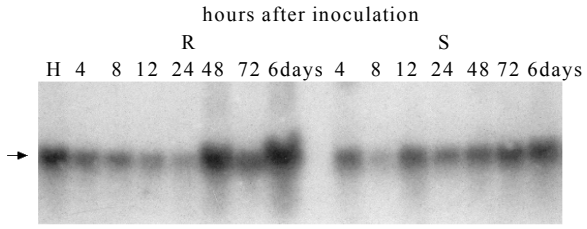
The temporal expression of the individual defense-related genes was investigated with northern analysis as revealed by the autoradiographs and by measuring regression analysis of the radioactive signal intensity against the specific time points.

PAL (Figures 15 A and B): In the first few hours, between 4-8 h period, there was a dramatic fall in PAL concentration in compatible reactions, compared to resistant plant. This was followed by the susceptible plants displaying a relatively slower rise in PAL activity, the peak concentration (~140 k O.D) not reaching as high as in resistant plants (~180 k O.D). In incompatible reactions, there was initially a gradual decrease in the expression of PAL between 4 to 24 h followed by a dramatic ascend between 48 to 144 h (6 d).

Chitinase (Figures 16 A and B): This gene probe showed a clear-cut difference between compatible and incompatible reactions. In resistant plants, there was a slight increase between 4 to 24 h (~15 k O.D.) followed by an upward trend at and up to 72 h (~105 k O.D.). The expression in susceptible plants remained close to a basal level (~25 k O.D.) i.e. similar to healthy control plants.

Lipoxygenase (Figures 17 A and B): At the beginning between 4 to 24 h, the expression level in both resistant and susceptible genotypes was similar (~200 and 175 k O.D. respectively), followed by a rise in susceptible (~240 k O.D.) and downward trend in resistant seedlings (~90 k O.D.) up to 72 h.

A



B

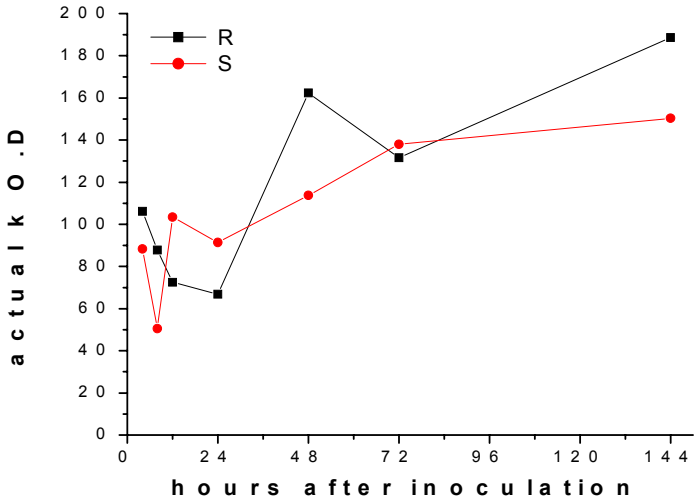
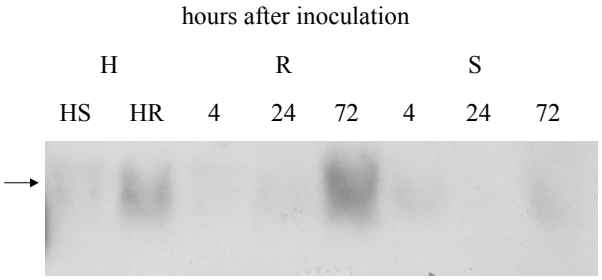


Figure 15 Time courses of changes in Phenylalanine ammonia lyase (PAL) transcript accumulation in pearl millet seedlings inoculated with *Sclerospora graminicola* zoospores. A: Northern blot analysis of PAL at the different time intervals. Lanes marked H = Control RNA from susceptible type, R = resistant, S =susceptible RNA. The arrow points out to the sizes of bands, 1.8 Kb. B: The graphical representation of the autoradiogram, x-axis: kilo O.D (Optical density - Background intensity / mm^2) considering the actual O.D of the test sample and y-axis: range of time intervals 0 to 144 hours after inoculation. The graphs were portrayed using ORIGIN 6.0 (Microcal Software Inc.). ▪ resistant inoculated, ● susceptible inoculated.

A



B

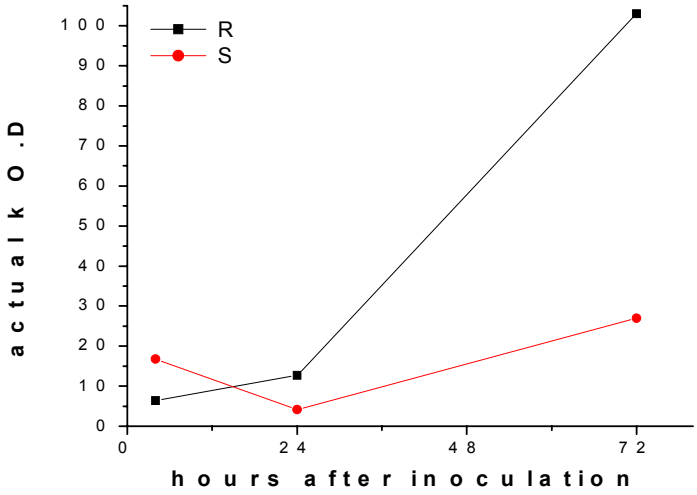


Figure 16 Time courses of changes in chitinase expression in pearl millet seedlings inoculated with *Sclerospora graminicola* zoospores. Lanes and experimental description as described for Figure 15, with the exception that HS = control RNA from susceptible type and HR = control RNA from resistant type. A: Northern blot analysis. B: Graphical representation, where x-axis: % O.D and y-axis: time intervals 0 to 72 hours after inoculation.

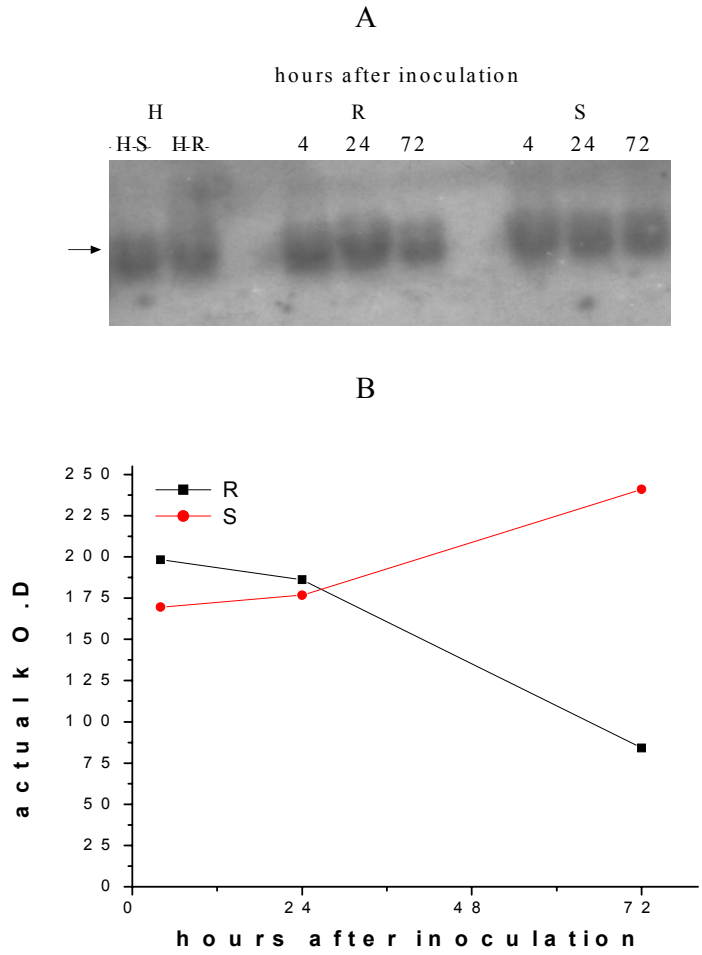


Figure 17 Time courses of changes in lipoxygenase expression in pearl millet seedlings inoculated with *Sclerospora graminicola* zoospores. Lanes and experimental description as described for Figure 15 with the exception that HS = control RNA from susceptible type and HR = control RNA from resistant type. A: Northern blot analysis. B: Graphical representation, where x-axis: % O.D and y-axis: time intervals 0 to 72 hours after inoculation.

4.7 Discussion

Current data indicate that a moderate percentage of base identity exists among the defense-related genes between heterologous species as revealed by BLAST searches from GenBank (Linthorst, 1991; McDowell and Dangl, 2000), and hence I considered that the experiments on cloned genes from other host systems could unfold preliminary patterns of gene activation. Many studies have focused on model systems like *Pyrenopeziza Brassicae* - *brassica* (Ashby, 1997), *Cladosporium fulvum* - tomato (de Wit et al., 1997) and *Fusarium graminearum* - wheat (Pritsch et al., 2000 and 2001) and have proved that

heterologous probes are helpful in providing an insight into host-pathogen interactions. The interaction between *Helianthus annus* and *Plasmopara halstedii* has revealed that there are different timings of specific RNA accumulation leading to plant protection and these timings have been used as markers for resistance (Mazeyrat et al., 1998). However, one has to anticipate differences between genomes and the interpretations are complicated in a system like mine, where both host (Brunken, 1977) and pathogen are out-breeding (Idris and Ball, 1984). Consequently, I used the key defense-related genes namely PAL, chitinase and lipoxygenase from established systems to elucidate the chain of pathogen mediated response in compatible and incompatible reactions of downy mildew - pearl millet.

PAL

The complex regulation of PAL by various stress stimuli has been reported in pea (Ralton et al., 1988), parsley (Kombrink et al., 1993), bean (Eastbrook and Sengupta-Gopalan, 1991) and tobacco (Liang et al., 1989). Cui et al., (1996) observed hypersensitive flecks and pigments after 20 h of infection while PAL mRNA accumulation was noted in 48 h in resistant plants. In my study on downy mildew of pearl millet, the first visual changes such as lesions on the leaves were observed 6 d (144 h) after infection, though the differential molecular expression was triggered within hours. The susceptible plants displayed a mild rise in activity while the trend was more remarkable in the 24 h to 6 d (144 h) period in the resistant plants. Bell et al., (1986) too observed a rise in PAL and chalcone synthase mRNAs in resistant tissue while susceptible tissues showed a decreased and delayed response. My results are in agreement with enzymatic studies by Nagarathna et al., (1993) who described an increase of PAL enzyme, 12 to 24 h after fungal inoculation in resistant reaction and a reduced activity in susceptible interaction. Cases of rise in PAL activity in resistant plants after 24 h were observed with some temporal differences in other systems such as potato-*Phytophthora infestans* (Henderson and Friend, 1979), whereas cowpea-*Phytophthora vignae* (Ralton et al., 1988) displayed a dramatic increase after 12 h.

The above results substantiate to some extent that in incompatible reaction, PAL expression is up regulated by downy mildew pathogen as an active defense mechanism of pearl millet. The increase in PAL activity can also be a downstream of hypersensitive response (McDowell and Dangl, 2000). Increase in PAL activity has been attributed to ethylene synthesis and free radicals generation leading to hypersensitive response at the site of infection, lignin deposition (Janshekar and Fiechter, 1983) and control of the

translocation of host nutrients thereby strengthening the plant against fungal growth. Lignin accumulation has been observed in infected resistant pearl millet seedlings (Nagarathna et al., 1993) and my studies on PAL, which is involved in lignin synthesis, support these changes.

Chitinase

Chitinase has been extensively studied in plants as a secondary hydrolase involved in defense. Its substrate chitin does not occur in higher plants but is present in the cell wall of many fungi. Chitinase assays in cucumber–*Colletotricum lagenarium* system exhibited a strong increase in activity in resistant young leaves, which restricted the spread of infection (Boller, 1987). Studies on tobacco-*Phytophthora* interaction demonstrated a rise in resistant plants between 12 to 16 h, followed by a gradual increase up to 96 h (Oelofse and Dubery, 1996), its highest activity reaching at 24 h after elicitation. A similar expression was observed in wheat–*F. graminearum* interaction with initial accumulation at 12 to 24 h after inoculation, which continued between 36 and 48 h (Pritsch et al., 2000). In resistant seedlings, I obtained an unambiguous rise of the chitinase transcript between 4 to 24 h period and a peak at 72 h, while in susceptible the levels of the transcript remained low, close to the basal concentrations. It has been reported that fungal infection can act as an independent elicitation of chitinase in bean, which exists as a small multigene family of approximately four members, at least two of which are differentially expressed during infection by pathogen (Broglie et al., 1986).

Lipoxygenase

In resistant genotypes, lipoxygenase has been observed to increase in early stages of germination followed by a subsequent decrease in wheat (Guss et al., 1968.) and rice (Ohta et al., 1986), giving them an advantage of vigor. My studies on susceptible and resistant cultivars of pearl millet showed similar changes of lipoxygenase in the 4 to 24 h period, followed by a slight upward trend in susceptible seedlings and diminished expression in resistant seedlings. The initial higher level of lipoxygenase in resistant seedlings is probably an indication of vigor, which could be due to stimulated electrolyte leakage and enhanced lipid peroxidation as observed in *Cladosporium fulvum* infected tomatoes followed by a decrease which gave way to recovery (Peever and Higgins, 1989). On the other hand, the susceptible seedlings showed initially a low level of lipoxygnease followed by an upward pulse causing a decrease of vigor due to excess electrolyte leakage and the seedlings could not recover. In pearl millet, Nagarathna et al., (1992) observed that

increases in lipoxygenase enzymatic activity in developing seedlings could be related to disease susceptibility of the genotype and decrease of resistance. To date, studies on lipoxygenase have indicated its role in seed germination, and in disease resistance, while its metabolism products have been found to have antimicrobial activities (Galliard and Chan, 1980). My studies at the lipoxygenase gene level support evidence to some extent to its function as a parameter to differentiate pearl millet cultivars for their resistance or susceptibility to downy mildew.

The changes in RNA levels of PAL, lipoxygenase and chitinase have shown that induced cultivar resistance (Vanderplank, 1984) is exhibited by pearl millet and one can expect parasite specific recognition processes (Heath, 1991). The results obtained support the view that plant defense is a multi-component dynamic system with different protective mechanisms having complementary roles in disease resistance. Numerous pathogen-inducible genes function synergistically in signal transduction, and demonstration of their functions will require a concerted application of biochemical, molecular and genetic approaches.

Comparison of changes in transcript to morphological observations: a speculative analysis

In this study, I explored the understanding of downy mildew by describing the early pathogenesis related gene changes during infection in relation to known morphological events largely based on the findings by Subramanya et al. (1983) and Singh S.D., ICRISAT, India (personal communication). Plants challenged with compatible pathogen isolates develop chlorotic lesions in 6 to 10 d, whereas this study of defense-related genes could detect changes at 4 h stage, coinciding with the reported histopathological modifications such as successful colonization and sporulation in susceptible varieties (Subramanya et al., 1983). Moreover, appresorium (fungal structure that helps the hyphae in the infection process) formation and penetration takes place after 4 h, while secondary vesicles are formed within 24 h leading to intracellular spreading of the systemic fungus, followed by intercellular mycelium in 48 to 96 h. Reduced host PAL and chitinase expression are known to coincide with such morphological changes of the pathogen and the timing of the RNA events of my study match the expression changes of these two genes. Cowpea – *Phytophthora vignae* showed fungal hyphal growth initially in resistant and susceptible epicotyls followed by a striking difference later between the two varieties, suggesting that resistance mechanisms are effective in inhibiting hyphal invasion after an

initial penetration period while in susceptible plants the hyphae spread and occupy the plant tissue (Ralton et al., 1988). *Solanum* and lettuce species exhibited lignin-like globules, containing phenolic compounds in resistant varieties on elicitation by *Phtophthora infestans* and *Bremia lacatucae*, respectively (Vleeshouwers et al., 2000).

Resistant pearl millet lines show increased number of papillae (structural thickening of plant cell wall against hyphae) and decreased haustoria (used by the fungus in absorption of nutrients from host cells) on pathogen challenge and *vice versa* in susceptible lines (Sharada et al., 1995; Thordal-Christensen and Smedegaard-Petersen, 1988), which can now be correlated to host enzymes like chitinase. Its remarkable expression in resistant seedlings potentially signifies the killing of advancing hyphae, thereby assisting in defensive combat.

Based on the above information, it appears that RNA assays are useful tools for quick and approximate screening of progeny in breeding programs and also for studying cellular responses. In my study, the incompatible reaction was differentiated by rapid changes in defense gene levels. Another study reported on infected pearl millet seedlings observed a 90 kD cell wall protein by SDS-PAGE whose absence or low concentrations signified resistance (Kumar et al., 1993).

Downy mildew affected plants exhibit leafy structures or 'green ear' in place of healthy panicles as they cannot flower (Brunken, 1977; Semisi and Ball, 1989). When I tested flowering related MADS box gene clones namely GLO and DEF (obtained from H Saedler Max Plank Institute, Cologne, Germany) to observe their involvement in the green ear disease at the seedling stage, no significant differences in transcript levels were noted. It is speculated that these changes take place in the later stages of the disease; approximately 30 - 60 d after inoculation, when flowering of the plant is affected. It will be hence interesting to find the involvement of the homeotic MADS box genes in downy mildew infection and specific localization of the enhanced transcript accumulation in the individual tissues of the panicle. Presently my group is using the pearl millet-downy mildew system to study different stages of infection at the microscopic, biochemical and molecular levels, particularly the early events that discriminate compatible and incompatible interactions for diagnosis.

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Chapter 5

Conclusion and Future Perspectives

Sclerospora graminicola (Sacc.) Schroet is an important constraint to sustainable pearl millet production in India and Africa (Singh et al., 1993). Previous reviews on downy mildews have concentrated widely on microscopic changes, description of compatible - incompatible reactions, resistance breeding and formulating strategies to use molecular approaches for the better understanding of this disease (Michelmore et al., 1986; Hash et al., 1997; Jeger et al., 1998).

With an attempt to gain a deeper insight into the pearl millet- downy mildew interaction, my thesis sketched the following aspects:

1) Study of diversity of the pathogen using isolates from different regions of India and analyzing the genetic relationships of the isolates based on AFLP fingerprinting.

2) Retroelement analysis of downy mildew with particular emphasis on the P5 element.

3) Use of DDRT – PCR and pathogenesis specific candidate gene approach to gain an understanding into plant – pathogen relationship.

5.1 Population dynamics of downy mildew of pearl millet

Sclerospora graminicola is a highly variable pathogen and previous studies by our group have explored adaptation of pathotypes to changed hosts over ten asexual generations using molecular approaches (Sastry et al., 1995, 1997 and 2001). Earlier studies by Ball et al. (1986; Semisi and Ball, 1989) have shown geographic similarity among West African collections of *S. graminicola* that were found to be more pathogenic than those from Niger, Senegal and Zambia (Werder and Ball, 1992). In my thesis work, I have studied the genetic diversity in the Indian pathogenic population using the AFLP fingerprinting approach and have demonstrated the presence of two clusters and their respective subclusters. There are several possible ways to explain this type of grouping in the population. Pathogen isolates having opposite mating ability as (+) and (-) strains to produce sexual oospores confirm the genetic relationship to an expected variation. In some cases, the genetic distance between the isolates reflects the physical distance between collection sites while in others differences in virulence characteristics are represented by wide genetic distance. The isolates having common host cultivars from which they have been collected also show distinct clustering in a common group and exceptions have revealed the typical heterogeneity of the out-breeding pathogen population. Classification of the host genotype specific isolates into two specific clusters supports the mechanism of virulence changes through asexual generations. Availability of sexual and asexual propagules could make the

process of change more rapid, causing new recombinants to establish themselves, thus increasing the probability of polymorphism. On a wider scale, the application of AFLP to detect genetic variation is particularly significant to select downy mildew isolates to screen breeding material for choosing resistant pearl millet and monitoring changes in *S. graminicola* in relation to changes in host. It is anticipated that fingerprinting profiles of different pathotypes and isolates of downy mildew can be used as diagnostic tools to formulate breeding strategies targeting resistance to local population and for monitoring and warning the emergence of new races.

As a future line of work, development of molecular markers linked to virulence will be of considerable importance in identifying such new recombinants. Further, these data can add to the knowledge of population structure and dynamics of downy mildew that has been generated in this thesis.

5.2 The P5 retrotransposon in downy mildew: putative role in plant – pathogen interaction

Retrotransposon-like elements bring about considerable genetic variability by transposition within the genome they harbor through spontaneous mutations. They help the fungal populations to adapt successfully to environmental conditions such as biotic (Moreau-Mhiri et al., 1996) and abiotic stress factors (Anaya and Roncero, 1996). One example among several others, of pathogenic retrotransposon mutating a host plant's gene thereby converting an incompatible interaction to a compatible one (Kearney et al., 1988), set the foundation of my studies regarding the presence of P5 retro element in the genome of downy mildew and its putative expression in compatible interaction between 7042S cultivar of pearl millet and downy mildew pathotype 5. A monomorphic distribution of P5 has been found among the 5 downy mildew isolates selected, a result similar to that obtained in *Fulvia fulva* (Mc Hale et al., 1989). Northern analysis using P5 element as a probe has revealed an amplified expression of the retro element and a band of size 1.8 kb in the infected susceptible cultivar that is absent in the infected resistant cultivar. This indicates an expression of the fungal retro element after systemically infecting its host. It is also possible that on infection, the plant's intrinsic P5-like retroelement is amplified, which could have a role in the compatible reaction and which is suppressed in resistant cultivars. As the fungus cannot be grown axenically, it has been difficult to quantify the specific differences in expression between plant and pathogen during the process of infection.

It will be interesting to explore retrotransposon-like elements for generation of molecular markers in downy mildew pathogen populations and also to distinguish compatible and incompatible interactions with pearl millet generated by their different genetic, structural and dispersion properties.

5.3 Differential expression of pathogenesis related genes

The cellular communication between the virulent pathogen and its susceptible host and also between an avirulent isolate and its resistant host cultivar is complex. Lack of full understanding of the infection processes by *S. graminicola* has hampered the development of rational control strategies. With technological advancement, the spatial and temporal differences in the expression of the myriad of molecules can be partly understood by studying the interaction at RNA level using Differential Display Reverse Transcriptase – PCR (DDRT-PCR). The DDRT – PCR products can be used to screen genomic and cDNA libraries to isolate gene families in heterologous systems or can be amplified by PCR using the respective degenerate oligonucleotide primers (Truesdell and Dickman, 1997). These genes could be putative defense-related genes and represent markers for compatible host – pathogen relationship. I have conducted the DDRT-PCR experiments and have used the northern analysis approach for unraveling the downy mildew-pearl millet relationship. The subtraction technique could reveal cDNA fragments with sizes in range of 400-600 bp using different primer combinations such as T_{12}MA-OPK7, T_{12}MG-OPC4, T_{12}MC-OPC4 and T_{12}MC-OPK3 in susceptible plant samples that were absent in healthy and sporangial tissues. The role of such fragments in signal transduction pathways as constitutive functional genes or induced biotic stress proteins will be interesting to examine.

Several proteins that are specifically induced by pathogens in one host species have related counterparts in many other species (Linthorst, 1991; McDowell and Dangl, 2000). With this idea, three well-established defense-related proteins namely PAL (Phenyalanine ammonia lyase), chitinase and lipoxygenase were used by me to study their expression over a time period to understand the interaction between pearl millet and downy mildew. I have observed a difference between susceptible and resistant cultivar reactions to infection, which was compared to the constitutive expression of the genes in healthy seedlings. From my study, it is clear that chitinase level is higher in resistant seedling than susceptible, while lipoxygenase levels decrease in comparison to susceptible seedlings of pearl millet upon pathogen infection. PAL levels have been found to increase in susceptible and resistant seedlings but the trend is stronger in resistant and this can be considered as the

effect of hypersensitive response. I have also measured the changes in the selected gene levels and have tried to relate them to known changes in microscopic symptoms after infection by downy mildew pathogen. Based on my study, it appears that the RNA assays can be used for quick diagnosis of cellular responses of the host and can aid a fairly accurate screening in breeding programs.

5.4 Future prospects

Because of the exceptional ability of pearl millet to tolerate drought, this crop is likely to play an important role in meeting the near future food grain requirement in regions, which are too arid for other crops like sorghum, wheat and maize. However, downy mildew may continue to be a major threat to pearl millet if economical methods to overcome the disease are not adopted (Burton, 1983). Employment of a mosaic of resistant cultivars and continuous monitoring of the pathogen populations to keep abreast with the virulent races will be essential for the profitable cultivation of pearl millet.

My research has explored some key findings on downy mildew and pearl millet and future efforts could be directed to:

1) Characterization of virulence genes and diagnosis of changes in pathogen populations as prior warning to farmers can be carried out using molecular markers including SSRs to aid in effective screening and control of downy mildew.

2) Identification of novel resistance genes in the host pearl millet to engineer defense against pathogen.

3) Development of finer procedures and reduction of time and expense of pyramiding genes for resistance to downy mildew, ergot, smut and tolerance to drought in pearl millet for increased production and higher yield potential.

The above strategies might help to restrict the development of virulent populations of *S. graminicola* for longer duration leading to stable cultivation of pearl millet. Emerging trends in rice and wheat are leading examples of attempting durable resistance to a broad spectrum of pathogens resulting in increased yield. In rice, heterosis has resulted in a yield advantage of 10-15 % while breeding the semi-dwarf lines with desired traits such as better grain filling, and introducing genes for disease and insect resistance are being tried to improve yield potential (Khush and Baenziger, 1998). Similarly, wheat cultivars are being bred for pathogen resistance, herbicide-resistance, tolerance to heat and cold and ability to withstand local environmental changes so that rotations involving wheat become more

flexible. Major breakthroughs that have occurred in the cellular and molecular biology of these crops have made them ideal models for biotechnology research of cereal crops. Future progress in pearl millet – downy mildew interaction can be made by forming feasible correlations to the control strategies in model crops, which would help in increasing the productivity and sustainability of pearl millet.

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List of Publications

1. **R. Singru, S. Sivaramakrishnan, R.P. Thakur, V.S. Gupta and P.K. Ranjekar (communicated and accepted, 2002)** Detection of genetic variability in pearl millet downy mildew (*Sclerospora graminicola*) by AFLP. Biochemical Genetics.

2. **R. Singru, V.S. Gupta and P.K. Ranjekar (communicated and accepted, 2003)** Identification of P5 retrotransposon like element in *Sclerospora graminicola* and contemplation of its role. World Journal of Microbiology and Biotechnology.

3. **R. Singru, S. Sivaramakrishnan, R.P. Thakur, V.S. Gupta and P.K. Ranjekar (communicated and accepted, 2003)** Differential expression of plant defense genes in pearl millet-downy mildew interaction. Indian Journal of Phytopathology.

4. **Sastry JG, Sivaramakrishnan S, RaoVP, Thakur RP, Singru RS, Gupta VS, and Ranjekar PK (2001)** Genetic basis of host specificity in *Sclerospora graminicola*, the pearl millet downy mildew pathogen. Indian Journal of Phytopathology 54 (3): 323-328.