MOLECULAR STUDIES ON GENETIC VARIABILITY AND PLANT-PATHOGEN INTERACTIONS IN PEARL MILLET DOWNY MILDEW (*SCLEROSPORA GRAMINICOLA*) PATHOGEN

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BY

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DECLARATION

Certified that the work incorporated in the thesis entitled "**Molecular studies on genetic variability and plant-pathogen interactions in pearl millet downy mildew (*Sclerospora graminicola*) pathogen**" submitted by Mr. **Jayanty S. S. G. M. Sastry** was carried out by the candidate under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

(P. K. Ranjekar)

Research Guide

INTER-INSTITUTIONAL COLLABORATIVE RESEARCH EFFORT

THIS WORK IS AN OUTCOME OF COLLABORATIVE RESEARCH PROGRAM BETWEEN

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&

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DEDICATED TO MY PARENTS

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AKNOWLEDGEMENTS

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J. S. S. G. M . Sastry

LIST OF ABBREVIATIONS

bp	base pairs
kbp	kilobase pairs
dCTP	deoxycytidine 5'-triphosphate
dATP	deoxyadenosine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
Tris	tris-hydroxymethyl amino methane
SDS	sodium dodecyl sulphate
EDTA	ethylene diamine tetra acetic acid
DTT	dithiothreitol
TAE	tris-acetate - EDTA buffer
TBE	tris-borate -EDTA buffer
TPE	tris-phosphate - EDTA buffer
MOPS	3[N-morpholino] propane sulfonic acid buffer
PBS	phosphate saline buffer
SE	standard error

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CHAPTER-I

AN INTRODUCTION TO DOWNY MILDEW HOST-PATHOGEN RELATIONSHIP AND ITS MOLECULAR CHARACTERIZATION

Many crop plants are susceptible to fungal pathogens resulting in considerable crop losses. These pathogens are continuously evolving in the face of selection pressure exerted by crop monoculture. Modern molecular approaches are increasingly becoming indispensable tools to study and understand population genetics and plant resistance to fungal pathogens. Still it is a long way for comprehensive understanding of all the processes involved in the relationship between host plant and pathogen, especially the highly specialized obligate biotrophic pathogens, such as the downy mildews.

A. Pearl millet - the host

Pearl millet [*Pennisetum glaucum* (L.) R.Br.], is an important cereal crop with its ability to grow on soils of marginal fertility in semi-arid tropical environments with an annual production of about 13 million tons (1). Pearl millet accounts for about 40% of the world millets production (2). It is thought to be originated in the Sahelian zone of West Africa (3) and probably domesticated along Southern margins of the Saharan Central Highlands 5,000 to 6,000 years ago (4). As a semi-arid crop, it is traditionally a component of the dryland system. No other crop has been found equal to or surpassing the dependability of pearl millet as a source of food for a large number of subsistence farmers that inhabit the semi-arid tropics (5). Pearl millet is mainly grown for grain and forage on about 15 million ha in Africa and 10 million ha in Asia (6). In India, it is the fourth most important cereal food crop and is chiefly grown in the states of Rajasthan, Gujarat, Maharashtra and Uttar Pradesh.

In USA, it is one of the best annual summer forage crop for drier regions and also grown in Australia and South America for the same purpose. Nutritionally superior to wheat and rice, pearl millet is commonly used to make unleavened. bread, thin or thick porridge or cooked like rice. It is of economic importance in India and in many other countries in Africa, including Senegal, Mali, Burkina Faso, Niger, Nigeria, Togo, Chad, Tanzania, Zambia and Mozambique (7).

Discovery of cytoplasmic male sterile (CMS) lines in pearl millet heralded the beginning of a successful hybrid breeding program in India. The introduction of CMS line Tift - 23A (8) from USA resulted in mass planting of potentially high yielding homogenetic F_1 hybrid cultivars over large areas in India. All the

hybrids in India were based on the Tift - 23A line, which was developed in the absence of downy mildew disease at Tifton, Georgia, USA. However, in India within few years of introduction, hybrids from this seed parent began to show susceptibility to the disease, and the Indian pearl millet hybrid program was severely affected.

B. Downy mildews - phytopathogenic fungi

Downy mildew fungi belong to the class Oomycetes. In Oomycetes, mode of parasitism ranges from saprophytic parasitism on plant and animal debris through facultative parasitism on fish, algae and higher plants to obligate parasitism on terrestrial plant hosts (9). Bruns *et al.* (10) have suggested that the Oomycetes are more closely related to diatoms and chrysophytes than to true fungi based on ribosomal DNA (rDNA). Phylogenetic analysis based on cytochrome oxidase gene sequences of *Phytophthora megasperma*, reveal that these fungi are more closely related to plants. There is now a growing consensus that Oomycetes should be considered as a phylum Oomycota, separated from true fungi in the kingdom of Chromista (11).

Downy mildews are distributed worldwide. *Peronosclerospora*, *Sclerophthora* and *Sclerospora* occur specifically in wet tropical regions whereas the genera of *Basidiophora*, *Bremia*, *Bremiella*, *Peronospora*, *Plasmopara* and *Pseudoperonospora* are characteristic for subtropical and temperate zones (Table1). Downy mildews can cause damage to the woody host plants (12) and also to members of Compositae (13). Some of the downy mildews primarily affect crop quality (e.g. *Bremia lactucae*, *Peronospora parasitica*, *Pseudoperonospora parasitica*, & *P. humuli*) while others affect yield (*Peronospora destructor*, *Pseudoperonospora cubensis*, and graminaceous downy mildews). Several downy mildew epidemics have been reported on different crops in different geographical regions (Table 2).

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Table 1 : Downy mildews, their hosts and distribution

Genus	Species	Host	Distribution	Ref.
Basidiophora	*B. entospora*	Compositae		(14)
Bremia	*B. lactucae*	Lettuce	World wide	(15)
Bremiella	*B. megaspora*	Viola		(16)
Peronosclerospora	*P. heteropogani*	Maize		(17)
	P. maydis	Maize	Asia & Australia	
	P. sorghii	Sorghum, maize & millet	Asia, Africa & America	"
	P. sacchari	Sugarcane & maize		"
Peronospora	*P. parasitica*	Brassicas	Europe, N.America & Africa	(18)
	P. hyoscyami	Tobacco	World wide	(19)
Plasmopara	*P. halstedii*	Sunflower	Europe, America & Africa	(20)
	P. manshurica	Soyabean	Asia N. America &Africa	(14)
	P. viticola	Grape vine	World wide	(21)
Pseudoperonospora	*P. cubensis*	Cucurbitaceae	World wide	(22)
	P. humuli	Hop	Europe, Japan & N. America	(23)
Sclerophthora	*S. macrospora*	Maize, rice & wheat	Africa America & South Europe	(17)
Sclerospora	*S. graminicola*	Pearl millet	Asia; Africa & N. America	"

The graminaceous downy mildews are members of *Peronosporaceae* (Oomycetes), responsible for major economic losses in important crops. Downy mildews belonging to *Peronosporaceae* affect at least 56 of the 330 plant families (24). All members of *peronosporaceae* are obligate biotrophic parasites (25) and they mostly infect and sporulate on undamaged host green tissue, mostly leaves. The two genera responsible for the most serious economic losses in graminaceous crops are *Sclerospora* and *Peronosclerospora*. Research on downy mildews is limited in comparison with other biotrophic fungi (e.g. rusts and powdery mildews). This may have been due to the difficulty of working with oomycetes pathogens under laboratory conditions, maintenance on living host plants, long term storage of viable inoculum, infection with oospores, diploid status of mycelium and spores, recovery of progeny from oospores and heterothalism (26).

Table 2. Downy mildew epidemics and affected areas.

Species	Area	Year	Reference
P. cubensis	Scandanavia		(27)
"	Slowenia	1988	(28)
"	Czechoslovakia	1985	(29)
P. sorghii	Nebraska	1987	(30)
P. radii	Israel	1987	(31)
S. macrospora	Khorasan (Iran)		(32)
S. graminicola	India	1970-76	(33)
		1983-84	(34)
		1987-88	(35)
P. tabacina	USA	1979-80	(36)

Sclerospora graminicola (Sacc), Schröt, the causal agent of downy mildew of pearl millet has a narrow host range infecting grasses in the tribe *Paniceae*, mainly pearl millet, foxtail millet, *Setaria spp.* and occasionally on maize (37; 38). *S. graminicola* is an obligate, diploid, biotrophic Oomycete. Michelmore *et al.* (39) have demonstrated that the pathogen is heterothallic and therefore pathogen is outbreeding.

Symptoms of fungal pathogen
Establishment of a successful biotrophic relationship relies upon maintenance of viable host cells and minimal damage to host tissue (40). Downy mildews establish a balanced relationship in which the host remains asymptomatic and severe damage to the host coincides with sporulation by the pathogen, under favorable conditions.
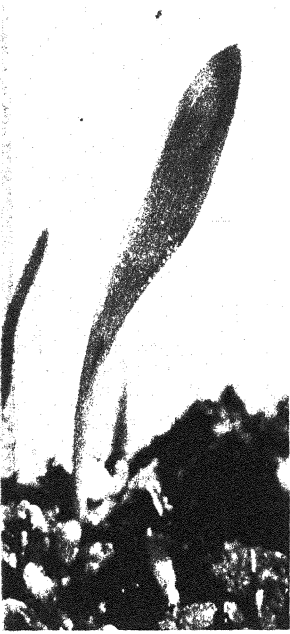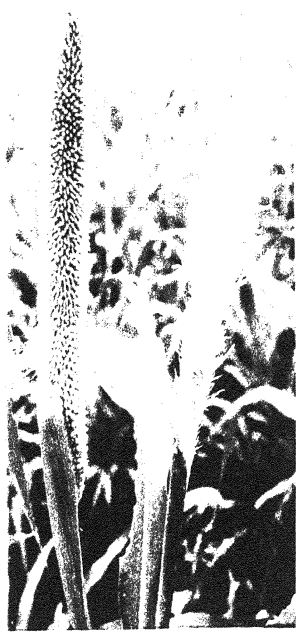
Fig.1a
Fig.1b

Fig.1a :Downy mildew infected pearl millet leaf showing downy symptoms.
Fig.1b :Healthy and malformed ear heads of pearl millet

Downy mildew fungus causes systemic infection. Some downy mildews cause local lesions usually from secondary inoculum infecting aerial parts of mature plants. Such lesions appear on leaves and develop in discrete patches. These chlorotic patches after sporulation by pathogen, degenerate and become necrotic. Most of the graminaceous downy mildews are exclusively systemic while *Sclerophthora rayssiae* on maize form only local foliar lesions (17).

S. graminicola causes systemic infection where the age of host plant is critical (17). The infection by the fungus may originate from either primary or secondary inoculum. Primary inoculum is due to thick-walled oospores, that remain in the soil and infect the underground parts of plants, mostly at the seedling stage leading to extensive colonization of roots, stems, leaves and often apical meristems. Systemic infection leads to stunting, abnormal growth and extensive chlorosis in pearl millet (41). Under humid conditions, infected leaves produce abundant sporangia on the abaxial surface (Fig.1a). If the environmental conditions are suitable, these thin-walled airborne sporangia spread to infect the neighboring fields as secondary spread (42). The extensive tillering and proliferation of leaves and floral parts is called "green ear" (Fig.1b). They do not bear panicle or produce panicle without grains. This depends on the severity of infection. Similar phyllody is seen in maize and other cereals caused by *Sclerophthora macrospora* known as "crazy top". Infected plants produce oospores that are transmitted on the seed surface in soil, by wind, or by water. Transmission of downy mildew by seed has been a subject of controversy (43), although mycelium has been found inside the seed (44).

Disease cycle

S. graminicola typically has an asexual cycle which results in short-lived, aerially dispersed sporangia and a sexual cycle which results in oospores.

a. Asexual cycle

This phase of life cycle is most sensitive to environmental conditions. Asexual sporulation occurs mostly on green host tissue. Sporulation is influenced by light, relative humidity and temperature. All downy mildew fungi require free water for germination and penetrate the host through stomata for infection. Sporangia of *S. graminicola* germinate while still on sporangiophore (45).

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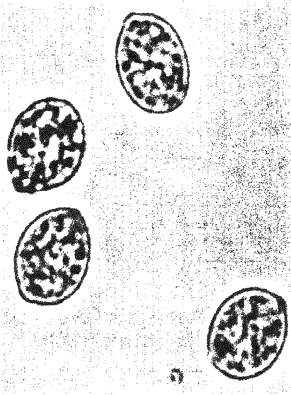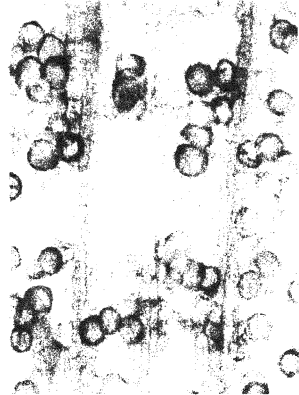
Fig.2a   Fig.2b

Fig.2a :Asexual spores (sporangia) of the downy mildew pathogen
Fig.2b :An infected leaf containing sexual spores (oospores) of the downy
        mildew pathogen.
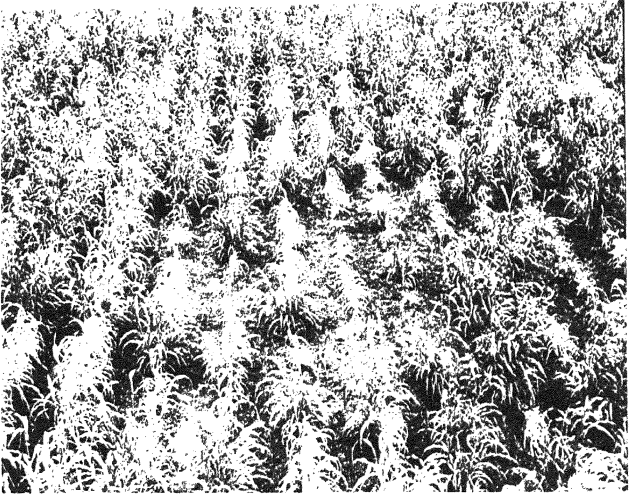
Fig. 2c : Downy mildew infected pearl
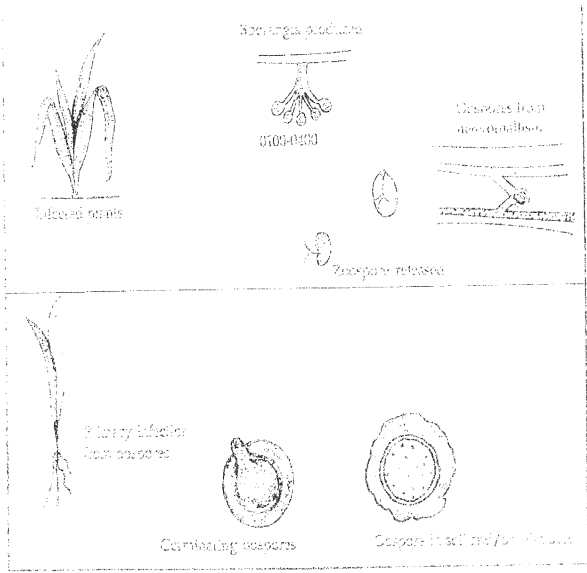
Sporangia produce

Infected plants

0100-0400

Sporangia from decapsillation

Zoospore released

Primary infection from zoospore

Germinating oospores

Oospore (oosed and/or in soil)

Fig.2. Disease cycle of *Sclerospora graminicola* the causal agent of downy mildew of pearl millet

In asexual phase, when relative humidity reaches to >95% at moderate temperatures (20-25^{0}C), the chlorotic areas on leaves produce abundant asexual spores, generally on the abaxial surface of the leaves giving them a downy appearance. Sporangia are hyaline, thin walled, ellipsoid and papillate (Fig. 2a). Usually sporangia are produced in the dark, and optimum sporangial production occurs at 20 to 25^{0}C and 95-100% RH (46). Sporangia may germinate directly by germ tubes or by releasing 1-12 zoospores (47). Zoospores germinate by germ tubes and retain their infectivity for longer hours at lower temperatures (48). The mycelium is predominantly intracellular and minimal host cell damage occurs. The host cell membrane is invaginated rather than ruptured by the haustoria.

b. Sexual cycle

S. graminicola is predominantly heterothallic and requires two mating types to form oospores (49). It is diploid for majority of its life cycle. Synchronous meiosis occurs in gametangia and fertilization occurs between single haploid nucleus from antheridium and single haploid nucleus of oogonium. The process of sexual reproduction initiates in antheridia and oogonia and culminates in the formation of oospores. Oospores are thick-walled resting spores that are formed in large number in the infected leaf tissue and malformed spikelets of the panicle. A mature oospore is brownish yellow and spherical (Fig.2b). Germination of oospores occurs directly by germ tube and indirectly by release of zoospores (46). Figure 2c shows downy mildew infected pearl millet field.

In case of lettuce downy mildew and pearl millet downy mildew, oospore infected seedlings were generated by sowing in oospore infested soil (50) but the percentage of infection obtained was very low. The erratic rate of recovery of sexual progeny from oospore has been often a limitation to the genetic analysis in downy mildews (26). Seed or soil borne oospores are, therefore, the source of inoculum for the recurrence of the disease in the following season. Schematic representation of asexual and sexual life cycle of downy mildew pathogen is shown in figure 3.

Epidemiology

In India downy mildew epidemics have occurred at regular intervals of once every three years on an average and during that period many new hybrids were withdrawn, because of their extreme susceptibility to downy mildew (Table 2). Downy mildews exploit aerial, soil or water borne avenues for infection. For successful host colonization the route of infection is important. Air borne sporangia mostly infect leaves resulting in localized lesions bound by veins. Infection to meristematic regions such as root and shoot leads to systemic invasion of host, and causes growth abnormalities. Systemic infection in pearl millet by *S. graminicola* is caused primarily by infection at the growing tips (51). Recently it has been shown that zoospores of *S. graminicola* can directly infect the inflorescence of pearl millet causing malformation of individual florets (52)

Alternatively downy mildew fungi may survive between seasons in perennial hosts or possibly on or in the seeds. Infection from seed is usually attributed to oospore contamination of the seed coat (17), however, transmission as mycelium in embryo tissue has been reported (43).

Control measures

Control measures have been designed to reduce the primary inoculum i.e. seed bearing oosporic inoculum and secondary spread of sporangia within and between fields. The methods used to control downy mildew can be classified into three categories: cultural, chemical and host plant resistance.

a. Cultural practices

All cultural control methods are aimed at manipulating the environment to the advantage of host and disadvantage of pathogen. Of the many known cultural practices, only rouging is strongly recommended for pearl millet downy mildew (53). This reduces the spread of the disease during the same season, and also oospore build up for the next season. Early sowing & transplanting are also suggested in pearl millet (54). Raghavendra Rao and Pargi (55) suggested a biological control by using mycoparasite *Fusarium semitectum* which grows on oospores of *S. graminicola*. However, these reports need confirmation and further studies on their practical application before their adoption.

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Time of planting is an important factor as it is related to soil temperature, humidity and other atmospheric temperature at a particular stage of the crop. Chahal *et al.* (56) reported the lowest downy mildew incidence in the early sown crop (June 29-July 3) in northern India. It may be possible that early sown pearl millet crop escapes the disease because of its growing in the weather conditions that are unfavorable to infection by pathogen. So it is obvious that more regional information on this aspect is needed which can be put to practical use. Crop rotation is also helpful in reducing the disease incidence as oospores are considered to be the major source of infection and they survive in the soil.

b. Chemical control

Chemical control for downy mildew has been attempted primarily by following two approaches: One is by treating seeds with fungicides to check the seed borne inoculum and second is by application of foliar sprays. The discovery and introduction of potent systemic fungicides with specific activity against oomycete plant pathogens, the control of foliar downy mildews improved considerably and made it possible to combat systemic seed- or -soil borne pathogens. The systemic fungicide (ridomil) metalaxyl (methyl)DL-(2, 6-demethyl phenyl)-N (2,methoxy acetyl) -N alaninate) has been used successfully to control downy mildew in pearl millet (57; 58; 59). Metalaxyl seed treatment is highly effective and economical but its foliar spray may not be practical and cost-effective for the poor subsistence farmers.

c. Host-plant resistance

Use of resistant cultivars is the most cost-effective method for the control of downy mildew. Recovery resistance is a phenomenon in which systemically infected plant out-grow the disease and produce healthy panicles. This trait has been detected in many pearl millet genotypes and has been increased through selection. This trait allows pathogen and host to coexist without affecting the yield (60).

C. Molecular approaches for pathogen analysis

Genome characterization

There are few reports on the characterization of genome of several important classes of fungi. For a long time classical and molecular genetic research on *Peronosporales* was rather limited. In Oomycetes, recently, detailed analysis of genome structure has been carried out in *B. lactucae* (61), and *Phytophthora spp.* (62). Our knowledge of chromosome number and genome size is very limited in downy mildews due to the small size of chromosomes. In phytophthora genus, the possible number of chromosomes is n=10 but there is a considerable variation from n=5 (*P. megakarya*) to n=11-15 (*P. megasperma*). The genome size of 2n to 4n isolates of *P. infestans* is 0.56 -1.11pg (62). McDonald and Martinez (63) have reported that *P. infestans* has much higher levels of repetitive DNA. In *Peronosporaceae*, karyotypes of *B. lactucae* and *B. parasitica* species have been determined so far. According to Michelmore *et al.* (45), it is expected that *B. lactucae* has n=7 chromosomes, haploid genome which contains $5x10^7$bp of DNA, and 65% of this nuclear DNA is repetitive. Low copy sequences are interspersed with repeated sequence in short interspersed fashion which is different from that described in other fungi (61).

In yeast and in other fungal systems, dispersed repetitive DNA sequences participate in a wide variety of genomic rearrangements (64). Hammer *et al.* (65) reported a family of highly polymorphic dispersed repeated DNA sequences MGR (*Magnoporthe grisea* genomic repeat) in *M. grisea*. These MGR sequences are routinely being used as fingerprinting probe for epidemiological studies (66).

Antequera *et al.* (67) analyzed methylation of DNA isolated from mycelia of 20 species of fungi representing 15 taxonomic families and found only 2 species in which there was detectable methylation of cytosine residues, as demonstrated by comparison of *Msp*I and *Hpa*II restriction patterns. The same authors reported that very little 5-mC was found in mycelial DNA from *A. nidulans* (68). Jupe *et al.* (69) reported that in fungal plant pathogen *Phymatotrichum omnivorum*, more 5-methylcytosine residues were found in DNA from dormant sclerotia than in the metabolically active mycelia of the fungus.

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Ploidy level

Study of ploidy levels in different fungal isolates are necessary since such differences may be underlying the variations in virulence and other characters noted among the populations. In Oomycetes fungi, the vegetative phase is mostly diploid or polyploid. Diploidy was postulated on the basis of cytogenetics in the case of *P. infestans* (70) and later Tooley & Therrien (71) found the existence of diploid, triploid and tetraploid isolates. In case of *C. albicans*, diploidy was confirmed by the comparison of DNA amount and complexity was determined by renaturation kinetics (72). Hulbert & Michelmore (73) confirmed the diploid status of oomycete *B. lactucae* by kinetic heterozygosity for RFLP markers. Later they found that *B. lactucae* was functionally diploid but there was evidence of polyploidy (73). The nature of ploidy can be important in establishing the level of genetic diversity in population and also provides clues to evolutionary relationships which help in the selection of species for genetic studies.

Retrotransposable elements

Retrotransposons, mobile genetic elements sharing some of the characteristics of retroviruses, have played an important role in the genome evolution of all eukaryotes and typically constitute about 5 to 10% of the genome (74). In retrotransposons, transposition is through the intermediate RNA molecule, which is subsequently reverse transcribed. The reverse transcriptase enzyme may have a significant impact on the genome because new insertion may induce both genetic and chromosomal mutations and may provide a mechanism for dispersing sequences in horizontal gene transfer. These elements include active and inactive derivatives of retrotransposable elements that are the most abundant class of dispersed repeats. A number of reverse-transcribed elements have been discovered by accident, and a variety of approaches have been developed for surveying the genome in search of such elements (75). Recently large number of retrotransposon - like sequences have been isolated from phytopathogenic fungi (76-79). A temperature-regulated retrotransoposon - like element was reported from *C. albicans* (80). Differential screening of powdery mildew fungus (*Erysiphe graminis*) using infected barley leaf cDNA library, yielded fungal clones that represent retrotransposons (81). The presence of such elements in

obligate biotrophs and their contribution towards host specificity and rapid adaptability to new cultivars is of much interest.

Genetic variability:

There has been a keen interest in devising methods that can be used to differentiate pathogenic and non-pathogenic strains, for better understanding of their epidemiology and population genetics and to develop control strategies. Fungi are often microscopic and have few phenotypic markers, such as vegetative compatibility, mating types or specific avirulence genes (82-84). Other biochemical criteria including isozymes (85-87) that can be used to differentiate between individuals in a population.

With the advent of highly polymorphic, molecular genetic markers, which are based on differences in DNA sequences has made it possible to conduct basic studies on population and evolutionary biology in fungi. These techniques include DNA hybridization methods, such as endogenous genomic and mitochondrial Restriction Fragment Length Polymorphisms (RFLP), DNA fingerprinting, and PCR - based Randomly Amplified Polymorphic DNA (RAPD) markers. These techniques have been widely used to estimate the genetic diversity in fungal pathogens. More than 70 species of zygomycetes, ascomycetes and dueteromycetes have been studied with the help of these approaches (88).

DNA fingerprinting has become one of the favorite methods for diagnostic and epidemiological studies of plant pathogenic fungi. Basically there are three kinds of DNA probes that have been used for fingerprinting studies in fungi. These include anonymous repetitive DNA probes derived from the fungal genome under investigation, minisatellite probes mostly derived from human or wild type M13 phage genome, and synthetic oligonucleotide probes complementary to simple repetitive sequences. The majority of DNA fingerprinting studies in fungi have relied on cloned genomic probes.

RFLP probes and RAPD markers have been successfully used to estimate the genetic diversity in fungal pathogens (89-91). In *Phytophthora* and some downy mildew fungi, only morphological, virulence and mating type markers were used. Isozyme and especially RFLP, RAPD polymorphisms were used for genetic characterization of *B. lactucae* (92; 73) and *P. halstedii* (93). Isozymes

were used for evaluation of interspecific differences in *Peronosclerospora* spp. (94). In *M. griesa*, genomic repetitive DNA sequences were used to detect polymorphisms (65). Human minisatellite probes 33.6 and 33.15 have been successfully used to distinguish pathotypes of *Colletotrichum gloeosporioides* (95). Commercially, many probes such as minisatellites M13, PV47 or simple repetitive oligonucleotides $(CA)_8$, $(CT)_8$, $(CAC)_5$, $(GTG)_5$, $(GACA)_4$ and $(GATA)_4$ have also been used in DNA fingerprinting. DNA fingerprinting using oligonucleotides, such as $(GATA)_4$, $(GTG)_5$, $(CA)_8$ and $(TCC)_5$, has been reported to detect variation among isolates of *Ascochyta rabiei* (96). In the case of filamentous fungi such as *Penicillium*, *Aspergillus* and *Trichoderma*, oligonucleotide probes, such as $(GATA)_4$ along with M13 minisatellite probe, have revealed informative DNA fingerprint patterns (97).

The development of PCR fingerprinting, using minisatellite and microsatellite core sequences as primers, has proved to be useful tool in detecting genetic variation (98). RAPD primers were also used in the case of *Collototrichum graminicola* (99), and *Puccinia striiformis* (100). Monastyrskii *et al.* (101) differentiated toxico-genic isolates of *Fusarium* by fingerprints.

D. Plant-pathogen interactions

Host specificity

Obligate fungal pathogens are host-dependent and highly adaptive, and evolve rapidly against the biotic and abiotic stresses. Downy mildews, as obligate biotrophs, have co-evolved with plant hosts over a long period leading to divergent forms of the pathogen adapted to different host taxa. Cultivar specificity is often determined by gene - for - gene interaction (102) and has been shown to control host-species specificity in many bacterial, viral and fungal host-pathogen systems (103; 104). The conventional genetic analysis indicates that specificity in the obligate fungal pathogens is determined by switching on the process of resistance. Newton and Crute (105) have summarized genetic evidence supporting the idea that gene-for-gene relationships might also control host species specificity in fungal plant-pathogen interactions.

Despite the progress achieved with certain bacterial and fungal pathogens, little is known about the determinants of specificity operating in biotrophic

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haustorium-forming pathogens, such as rusts, powdery mildews, and downy mildews. Pathogens adopt various kinds of mechanisms to generate specificity. Depending on compatibility and incompatibility factors between pathogens and hosts, the host range of a pathogen can be broad or narrow (103). There are different mechanisms to determine specificity involving different plant - fungus interactions. Newton & Crute (105) mentioned that there might be two distinct types of genetic regulation of specificity, based on active or induced resistance and passive or constitutive resistance. Mechanisms of induced resistance are predominantly in biotrophic host-pathogen association (106).

One mechanism of specificity in *Cochliobolous* and *Alternaria* is through fungal-host-selective toxins and plant detoxification mechanisms (107). The other strategy which is analogous to toxin producing fungal pathogens, is also followed by plants such as specificity through plant fungitoxins and fungal detoxification mechanisms (108-111).

Cloned avirulence genes have been used to detect the presence of matching *R* genes in host and nonhost plants (112; 113). Cultivar-specific fungal avirulence genes in case of *C. fulvum*, *Rhynchosporium secalis* and *M. grisea* have also been isolated. In *C. fulvum*, two avirulence genes *avr4* & *avr9* have been cloned and both elicit hypersensitive response on tomato cultivars carrying the complementary resistance genes *cf-4* & *cf-9* (114; 115). The *NIP1* avirulence gene from *Rhyncosporium secalis* elicits defense reactions in barley plants possessing the resistance gene *Rrs1* (116). Cultivar specificity has been identified in *M. grisea* at subspecies level determined by avirulence gene *avr2-yamo* gene on Yashiromochi rice cultivar (117).

In *B. lactucae*, one of the 61 restriction fragment length polymorphic (RFLP) loci is apparently linked to avirulence locus *Avr 6*, providing a potential starting point for chromosome walking to the avirulence gene (92). Vasanthi *et al.* (118) have reported the identification of *S. graminicola* cell wall protein present in pathotypes which are pathogenic on pearl millet hybrid HB 3, conferring host specificity. The cloning of resistance and avirulence genes will allow investigations on biochemical basis of specificity, mechanism of variations in resistance and virulence and development of novel strategies for disease control.

Plant responses to infection

In response to pathogens, plants set a large array of defense mechanisms. Plants exhibit natural resistance to pathogen attack by a combination of constitutive and induced defenses (119). They encode enzymes of polypropanoid pathway involved in the synthesis of antimicrobial phytoalexins, enzymes with hydrolytic activities, toxic compounds and enzymes that degrade cell wall proteins as part of constitutive defense reactions (119). Several microbial or fungal proteins (120-122) and oligosaccharides of either plant or fungal cell wall with elicitor activities have been characterized (123; 124). Different types of pathogens, such as facultative and biotrophic types, probably have different ways of exploiting their hosts and will therefore, expose different molecules of their respective host plants during pathogenesis.

It was known that peroxidase activity was associated with resistance of host plants and was used as a marker for resistance breeding. This phenomenon was studied in some downy mildew interactions (125). Nagarathana *et al.* (126) found good correlation of resistance to *S. graminicola* in pearl millet with lipoxygenase activity, while Sreedhara *et al.* (127) discovered peroxidase having a role in resistance to the same pathogen.

Recent studies employing targeted gene disruption mutants have revealed that cutinases (128-130), xylanases (131; 132) and other degradative enzymes (133-135) have no effect on pathogenecity (136). Knock-outs of candidate genes have provided evidence for involvement of specific signal transduction pathways in fungal pathogenesis. Studies in *M. grisea* have revealed that cpk A or pmk1 kinases participate in signal transduction pathways that are specific for pathogenesis (137; 138). The molecular mechanisms leading from plant/pathogen interaction to the development of the defense reaction are not well known. However, a number of resistance genes whose products may be involved in plant defense have been isolated recently. Resistance to pathogen involves a specific recognition between a resistant plant and pathogen. The specificity of this process is often determined by the product of plant resistance (*R*) gene and matching pathogen avirulence gene (102). The plant *R* genes seem to encode for receptors that interact directly with elicitors produced by pathogen *avr* genes (139). Recently, several *R* genes conferring race specific resistance

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have been cloned by positional cloning or transposon tagging (140-142) from several crop plants. These *R* genes, despite their origin from different plant species and their divergent specificity to viral, fungal or bacterial pathogens, share several common features (Table 3). Structurally, a nucleotide binding domain (P loop) and additional motifs of unknown function are conserved near their N-terminal regions. A region of leucine-rich repeat (LRR) of variable length and content is present at their C terminus which may play role in protein-protein interactions (143; 142) and protein phosphorylation, mediated by kinase domain (144) may play a role in pathogen recognition and activation of defense signals. The striking degree of structural and functional pathways suggests that they have co-evolved from an ancient defense signaling mechanism (145). Several *R* genes have been shown to belong to large, clustered gene families (146). However, detailed genomic distribution of these multigene families is not yet known. A major task in plant pathology now is to understand the precise nature of the *R* gene-mediated recognition events and consequent signaling processes involved.

Recently to study the underlying mechanisms in the process of infection and to gain more insight into the signaling process between host and pathogen, Differential Display RT-PCR is the method of choice. One of the principal advantages of differential display is that it permits the simultaneous identification of genes that are up regulated as well as down regulated. There are reports of using this technique to isolate mRNA species in host plants expressed in response to fungal infection and other abiotic stresses. In peas, Truesdell & Dickman (147) have reported the differential expression of SRG1 and SRG2 alfalfa cDNAs with the invasion of fungal pathogen *Colletotrichum trifolii*. Benito *et al.* (148) have successfully used this technique to study plant-fungal interactions. Three new cDNA species of *Botrytis cinerea* were isolated using DDRT-PCR whose expression was enhanced in tomato host plant. Significant advances have been made in relation to the population genetics in downy mildew fungi especially *Bremia*, *Phytopthora* and *Sclerospora* species but over all progress in this class of fungi is slow. Mapping avirulence genes in pathogen using molecular markers is feasible and it needs more attention. The successful genetic transformation of a few culturable oomycetes is another significant

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advance (149) but suitable cloning vectors incorporating promoters from downy mildews need to be constructed and these approaches can facilitate isolation and functional analysis of avirulence genes.

Molecular markers for tagging disease resistance genes

Most genetic studies on downy mildews have been aimed at breeding for disease resistance. The degree of knowledge on host resistance is closely linked with the search for effective sources of resistance. In pearl millet, major gene and race specific resistance to *S. graminicola* have been reported (150).

The ability to map genes in host plants, contributing towards various complex traits with accuracy for plant breeding applications, has recently been possible through the development of comprehensive molecular maps. Molecular markers such as RFLP, RAPD, and SCAR have been used for construction of comprehensive linkage maps in many crops. These molecular linkage maps facilitate the mapping of genes for complex agronomic traits. Molecular markers allow the genome to be broken into small segments so that the contribution of each of the smaller segments of chromosomes in a complex trait can be determined which is known as QTL analysis. QTL mapping does not require homogenous lines of the pathogen to be isolated as results can be obtained even with a variable pathogen population. Molecular markers will help in effective deployment of host resistance genes to provide more stable resistance and marker assisted selection will allow the production of a range of genotypes that will allow the durability of different resistant gene combination/deployment strategies. In pearl millet QTLs for resistance to *S. graminicola* from India, Nigeria, Niger and Senegal have been mapped (151).

Table 3: Cloned plant disease resistant (R) genes

R gene	Plant	Pathogen	Avr gene	Structure	Location	Ref.
Cf-2	Tomato	*Cladosporium fulvum*	Avr2	LRR TM	Transmembrane	(146)
Cf-9	Tomato	*C. fulvum*	Avr9	LRR TM	Transmembrane	(152)
Hs1	Sugar beet	*Heterodera schachti*	?	LRR TM	Transmembrane	(153)
Xa21	Rice	*Xanthomonas oryzae*	?	LRR TM PK	Transmembrane	(154)
Pto	Tomato	*Pseudomonas syringae*	avrPto	PK	Cytoplasmic	(155)
L6	Flax	*Melamspora lini*	?	TIR NBS LRR	Cytoplasmic	(156)
N	Tobacco	*Tobacco mosaiac virus*	Replicase	TIR NBS LRR	Cytoplasmic	(157)
RPP5	Arabidopsis	*Peronospora parasitica*	?	TIR NBS LRR	Cytoplasmic	(158)
RPM1	Arabidopsis	*P. syringae*	avrRPM1 avrB	LZ NBS LRR	Cytoplasmic	(159)
RPS2	Arabidopsis	*P. syringae*	AvrRpt2	LZ NBS LRR	Cytoplasmic	(160; 161)
12C-1	Tomato	*Fusarium oxysporum*	?	NBS LRR	Cytoplasmic	(162)

Leucine - rich repeats (LRR); Leucine zipper (LZ); nucleotide -binding
site (NBS); Protein kinase (PK); Trans membrane domain (TM);
sequence similar to the cytoplasmic domains of Toll and IL-1R (TIR)

Genesis of thesis

The obligate fungal parasitism is highly evolved in *S graminicola*, which can be used as a model system that offers an interesting opportunity for studying biotrophic plant diseases. The present thesis work is aimed at the following aspects:

1. Oomycetes ranges from saprophytes of plant animal debris to obligate parasites of terrestrial plant hosts (9). A very high percentage of repetitive DNA is reported in *Bremia lectucae* (61), which is more characteristic of slime moulds than filamentous fungi. So attempts were made to analyze repetitive sequences, transposable elements, methylation status and ploidy level in *S. graminicola* fungal population.

2. Study of variation among and within different populations from different geographic areas will show relatedness of these populations and will help us in the development of strategy for deployment of known resistance genes. Thus a resistant gene will only be deployed in areas where specific avirulence to that gene is present. In *S. graminicola*, a study was undertaken to analyse different populations collected from within a specific area and also from different geographic areas, to devise strategies and monitor the emergence of new virulent races using DNA fingerprinting technique.

3. Obligate fungal pathogens are known for their adapatability to overcome biotic and abiotic stresses. Several sources of resistance have been identified, however, these have frequently been rendered ineffective by variability in the pathogen population. The pathogen is cultivar specific and highly adaptive which prompted us to study the adaptability and pathogenecity on new cultivars.

4. This obligate fungus does not macerate host tissue and release toxins. It effects the hormonal changes in host plant and transforms panicle into leafy like structure. So in order to get better understanding of the host-pathogen . interaction, the process of infection was studied in the infected seedlings by looking at the differentially expressed RNA species.

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CHAPTER-II

CHARACTERIZATION OF THE GENOME OF *SCLEROSPORA GRAMINICOLA*, THE CAUSAL FUNGUS OF DOWNY MILDEW OF PEARL MILLET

Introduction

Until recently, the downy mildew disease caused by *S. graminicola* has received very little attention due to difficulty of maintaining the isolates on the living host plants under controlled environments and long term storage of viable inoculum. The diploid status of the mycelium and spores, heterothallism and the difficulty of recovering progeny from oospores make the genetic studies of pearl millet downy mildew difficult (26). The pathogen is highly variable and several host genotype-specific pathotypes have been reported (150).

An indepth knowledge of the genetics of host-pathogen interaction is essential not only for understanding the mechanism of variability in the pathogen population, but also and for development of strategies to control the disease. We have carried out genome analysis of *S. graminicola* with reference to the presence of repeat elements and retrotransposons.

Materials and Methods

Fungal material

S. graminicola pathotypes were maintained on seedlings of selected pearl millet genotypes by repeated inoculation with asexual spores in isolation chambers in a greenhouse. Genome analysis of six pathotypes namely Path-1 (NHB3), Path-2 (BJ 104), Path-3 (MBH-110), Path-4 (852B), Path-5 (700651), and Path-6 (7042 S) (150) was carried out. These pathotypes were from Patancheru population maintained on six different pearl millet cultivars asexually in a greenhouse.

Production of sporangia

The seeds of pearl millet cultivar were surface sterilized with 2% NaOCl for 5min and washed thoroughly with sterile distilled water. About 25 seeds were then sown in a pot containing sterilized potting medium (red soil, sand and farm yard manure in ratio of 1.5:1:1) and kept in green house at 30 ^{0}C for germination. Inoculum was prepared from downy mildew infected leaves which were collected and washed under running water with cotton web to remove the old sporangia. These infected

leaves were kept in moist chambers in an incubator at 20 ^{0}C for 6hrs under dark for sporulation. Sporangia from sporulated leaves were washed by brush into cold water and diluted to a concentration of 10^{-5}/ml for inoculation. Seedlings at first leaf stage were transferred from a green house to inoculation chamber and were inoculated either by spraying the inoculum or by putting a drop of inoculum in the leaf whorl. The infected seedlings were incubated for 24hrs at 20 ^{0}C and high humidity (90% R.H). They were then transferred to a greenhouse to maintain at 25 ^{0}C and 70% humidity (R.H) and were allowed to grow for 20 days for sporulation of infected leaves. The sporangia were collected in cold sterile water and pelleted at 10,000rpm for 10min in Sorvall centrifuge at 4 ^{0}C. The pelleted spores were stored at -70 ^{0}C and used for DNA extraction.

Fungal DNA isolation

The efficient disruption of cell wall is one of the critical steps during fungal DNA isolation. Fungal cell walls contain chitin and are often highly resistant to mechanical forces. A large number of methods to isolate fungal DNA have been described based on protoplast isolation using specific mixtures of cell wall degrading enzymes. For example, Zymolases are used to prepare protoplasts from which DNA can be extracted (163; 65). The other methods make use of detergents mostly CTAB, a DNA binding detergent and SDS to break the cell walls and organelle membranes (164; 165; 166). Modified method of Dellaporta *et al.* (167) has been found to be the best to isolate DNA from spores of *S. graminicola*.

Freezing spores in liquid nitrogen prior to grinding with mortar and pestle is an essential step in DNA isolation. The next step is to employ SDS to disrupt cell membranes and release DNA into the extraction buffer. The DNA is protected from endogenous nucleases by EDTA which chelates Mg ions that act as a cofactor for most nucleases. Chloroform is used to denature and separate protein from DNA. High concentration of NaCl in high salt $T_{50}E_{10}$ (10mM Tris-HCl, 1mM EDTA, pH 7.4) is mandatory for DNA to remain in sodium form and 70% ethanol removes residual SDS and salts. It is essential to remove RNA which is also precipitated

alongwith DNA and may interfere with spectrophotometric estimation of DNA and restriction endonuclease digestions.

The following protocol was used to isolate DNA from *S. graminicola*: Sporangia were harvested from sporulating infected leaves in ice-cold sterile distilled water and the suspension was centrifuged at 7000rpm for 20min at 5 ^{0}C in Sorvall RC 2C centrifuge. Approximately 2g (dry weight) of sporangial pellet was used to isolate genomic DNA. The sporangial pellet was powdered in liquid nitrogen in a mortar and pestle. The powder was extracted with 5 volumes of extraction buffer (50mM Tris-HCl, pH 8.0, 20mM EDTA, 0.5M NaCl and 1.0% SDS) and kept at 65 ^{0}C for 20min. An equal volume of phenol:chloroform (1:1) was added to the slurry, mixed gently and centrifuged for 10min at 10,000rpm in a Sorvall RC 2C centrifuge at 4^{0}C. The aqueous layer was removed and an equal volume of chloroform:Isoamyl alcohol (24:1) was added, mixed well and centrifuged for 10min at 10,000rpm for 10min at 4 ^{0}C. The aqueous phase was once again removed and nucleic acids were precipitated by adding 0.6 volumes of isopropanol. DNA was spooled with a glass rod, washed twice with 70% ethanol and suspended in $T_{50}E_{10}$ buffer. The DNA solution was treated with RNase (50 µg/ml) at 37 ^{0}C for 2hrs. An equal volume of phenol : chloroform was added to the solution at the end of incubation, mixed well for 5min and centrifuged in a microfuge (Eppendorf USA). The aqueous phase was transferred to another tube and DNA was precipitated by adding 2.5 volumes of chilled absolute ethanol. The DNA pellet was washed twice with 70% ethanol, vacuum dried and resuspended in $T_{10}E_1$ buffer (10mM Tris-HCl, 1mM EDTA, pH 7.4).

Plasmid DNA isolation

Bacterial plasmids, self replicating, circular extrachromosomal DNA molecules, are commonly used as vectors in recombinant DNA work. All plasmids contain 3 features, namely a replicator, a detectable marker and a cloning site. The detectable marker is usually a dominant gene encoding resistance to some antibiotic. The cloning site is a restriction endonuclease cleavage site into which foreign DNA can

be inserted. The high copy number plasmids exist in more than 20 copies per cell and their replicative mechanisms is independent of bacterial cell division.

Isolation of plasmid DNA from bacterial cells is essential for the analysis of recombinant clones. The alkaline lysis procedure (168; 179) is the most commonly used method for plasmid isolation. This method exploits structural differences between chromosomal and plasmid DNA. Bacteria are lysed by treatment with a solution containing SDS and NaOH where SDS denatures bacterial proteins and NaOH denatures chromosomal and plasmid DNA. The mixture is neutralized with potassium acetate, causing the covalently closed plasmid DNA to reanneal rapidly. Most of the chromosomal DNA and bacterial proteins precipitate with SDS which form a complex with potassium acetate and are removed by centrifugation. The reannealed plasmid DNA from the supernatant is then concentrated by ethanol precipitation.

In the present work, a single recombinant colony from the master plate was incubated in 1ml medium containing ampicillin (100µg/ml). This was grown to saturation at 37 °C overnight at 175rpm and the culture was pelleted in a 1.5 ml eppendorf tube. The pellet was suspended in 100ul GTE buffer [50mM Glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA (pH 8.0)],and kept on ice for 10min. For lysis of bacterial cells 200ul of lysis mix (0.2N NaOH & 1% SDS) was added. After 10min 150ul of 7.5M ammonium acetate (pH 5.2) solution was added, the solution was mixed well and kept on ice for 10 more minutes. The solution was centrifuged for 10min at 10,000rpm to pellet chromosomal DNA and cell debris. The plasmid DNA from the supernatant was precipitated with 2.5 volumes of ethanol and kept at room temperature for 10min. This was followed by a spin at 10,000rpm at4 °C for 10min, to pellet plasmid DNA and RNA. The pellet, washed with 70% ethanol and dried under vacuum, was dissolved in $T_{10}E_1$ buffer. The plasmid was treated with · 1ul (10mg /ml) of RNAse (DNAse free) at 37 °C for one hour to remove RNA. It was followed by phenol:chloroform treatments to remove RNase. This method was scaled up accordingly to isolate large amounts of plasmid DNA.

Restriction enzyme digestion of DNA

The restriction endonucleases recognize short DNA sequences and cleave double stranded DNA at specific sites. Genomic libraries of complex eukaryotic genomes can be generated by digesting the DNA with a restriction enzyme and ligating with a suitable vector cut with the same restriction enzyme. Restriction enzymes are extremely useful to construct restriction maps and to study the organization of specific gene sequences with the help of Southern hybridization.

In a typical digestion experiment, 1-2μg of DNA is incubated with a suitable restriction enzyme (5-10U/μg of DNA) mostly at 37 ^{0}C or at the specific temperature required for a particular enzyme. The salt concentrations are for maintaining optimum restriction enzyme activity according to the supplier's recommendations. Optimum enzyme concentration is used to avoid non-specific (star) activity of restriction enzymes. Control reactions are performed using commercial lambda DNA digested with respective restriction enzymes to check reaction conditions.

Various restriction enzymes such as Alu*I*, BamH*I*, Bgl*I*, EcoR*V*, HaeIII, Hinf*I*, HindIII, Hpa*II*, Msp*I*, Pst*I*, Pvu*II*, Sal*I*, Sma*I*, and Taq*I* were used to digest genomic DNA of *S.graminicola*. About 8-10μg of fungal DNA was digested with 40-100U of the restriction enzymes, according to supplier's instructions (Amersham, UK and New England Biolabs, USA). Methylation sensitive isoschizomeric enzymes such as Msp*I*, Hpa*II*, Mbo*I*, Sau3A*I* and Dpn*I* (Amersham, UK) were also used. The digested DNAs were separated on 1.0% agarose gels by electrophoresis in TPE buffer (90mM Tris-phosphate, 2mM EDTA, pH 7.5) followed by their transfer to nylon membranes by Southern blotting.

Agarose gel electrophoresis

Agarose is a linear polymer whose basic structure is composed of D-galactose and 3,6-anhydro L-galactose. Electrophoresis through agarose gels is a standard method used to separate, identify and purify DNA fragments. The DNA in the gel stained with a fluorescent intercalating dye-ethidium bromide is visible on exposure to UV light. Agarose gels have a lower range of resolving power than polyacrylamide gels

but have a greater range of separation. DNAs of 200bp to 20kb in length can be separated on agarose gels of various concentrations. In general agarose gels in TAE or TBE buffers are used to separate double stranded DNA. To analyze single stranded DNA, agarose gels in alkaline buffers (NaOH/EDTA) are used.

In our studies, genomic DNA digests were analyzed on 1% neutral agarose horizontal slab gels in TAE (40mM Tris acetate, pH 8.0 and 1mM EDTA, pH 8.0) at constant current of 20-40mA for 12-36hrs. Alternatively, TPE buffer was also used. After gel electrophoresis, the gels were stained in dark with ethidium bromide (1μg/ml) and were visualized on UV transilluminator (UV products, USA) with wavelength of 302nm and photographed with 35mm SLR camera using red filter on 135/36B/W ORWO 125 ASA film.

Southern blotting

Electrophoretically separated DNA fragments are generally transferred to membrane support as described by Southern (170). DNA is digested with restriction enzyme and the resulting fragments are separated according to size by agarose gel electrophoresis. The DNA is denatured in situ and transferred to a solid support such as nitrocellulose or nylon membrane. Although nitrocellulose membrane was initially used for transfer of DNA, it was not suitable for recurring use, because of its brittle nature and hydrophobic interaction with DNA. Nylon membranes are therefore, preferred to nitrocellulose for Southern blotting. Nucleic acids bind irreversibly and covalently to nylon membrane which can withstand repeated hybridizations. Specific nucleic acids that are immobilized on the membrane can be detected by hybridization of membrane bound DNA or RNA with radioactive or nonradioactive labeled probe. In the present work, the transfer of DNA from agarose gels to Hybond (Amersham) membranes or Nytran membranes was carried out using vacuum blotting apparatus (Pharmacia LKB Vacu Gene XL). The gel was placed on the vacuum blotting apparatus on top of the Hybond membrane and a pressure of 55psi was maintained in the vacuum pump. The gel was treated with 0.25N HCl for 10min for depurination of DNA and denatured in 1.5M NaCl and 0.5N NaOH for 15min. Neutralization of the gel was done in 1M Tris, pH 7.4 and

1.5M NaCl for 15min. The transfer of DNA onto the membrane was carried out for one hour in 20x SSC [(3M NaCl, 0.3M Sodium Citrate(pH. 8.0)], after which the membrane was rinsed in 2x SSC, air dried and baked for 2hrs at 80 ^{0}C.

Multiprime labeling

The labeled DNA probe when hybridized to unlabeled DNA strand on the membrane gives a signal on the autoradiogram depending on its sequence homology with the unlabeled DNA. The efficiency of such hybridization experiments depends on many factors such as salt concentration, temperature and the specific activity of radiolabeled probe. In the present experiment, DNA cloned in plasmid vector was labeled with [α-^{32}P]-dCTP by random prime labeling method of Fienberg & Vogelstein (171; 172). This method is based on the annealing of the mixtures of all possible combination of hexanucleotides to the DNA to be labeled at different positions on the template followed by extension by Klenow fragment of *E.coli* DNA polymerase-I in the presence of [α-^{32}P]-dCTP and other dNTPs to generate radioactive probe uniformly labeled on both the strands. Klenow fragment essentially catalyses the addition of nucleotides in a 5'-3' direction to the primer annealed to the template resulting in a phosphodiester bond formation between the nucleotides with the release of pyrophosphate. Since input DNA serves as a template and remains intact during the reaction, it is possible to label minimal amounts of DNA (10ng) to a high specific activity (10^8 cpm/μg DNA).

25-50ng of the probe DNA in a suitable volume of $T_{10}E_1$ was denatured by boiling for 10min and was immediately chilled on ice. To the denatured fragment, following reagents from multiprime labeling kit (BARC, India) were added : 5μl of primer, 5μl of labeling buffer, 4μl of each of dATP, dGTP, dTTP, 5μl (50μCi) of [α-^{32}P]-dCTP (specific activity 3000 Ci/m mole, BARC, India) and 2μl Klenow fragment (4U) (173). Final volume was made to 50μl with sterile water. Reaction was carried out at 37 ^{0}C for 2hrs and terminated by adding 10x stop dye (10% SDS, 125mM EDTA, 0.25% Bromophenol blue) to a final concentration of 1x to prevent background or non specific hybridization. The probe was separated from unincorporated labeled nucleotides by using spun column chromotography. A 1.5ml

microfuge tube was packed with sephadex G-50 to the level of 1ml by centrifugation in table top microfuge (Remi, India) at 1000rpm. The entire mixture containing labeled probe and free nucleotides was loaded on this exclusion column and spun at 1000rpm for few seconds. This was repeated thrice since the exclusion volume of the column was around 300-400μl. After spinning, the probe eluted in a volume of about 400μl and was used for hybridization.

Southern hybridization

To prevent the non-specific binding of the probe on to membranes, Southern blots were prehybridized for 2hrs at a particular temperature in heat sealed plastic bags containing the following mixture: 5x SSPE (3M NaCl, 0.3M Sodium citrate and 0.02M EDTA), 0.1% SDS, 5x Denhardt's solution (0.1% Ficoll, 0.1% Poly vinyl pyrrolidone) and 0.2x BLOTTO (1x BLOTTO is 5% defatted milk powder in water). When hybridization was carried out at 42 ^{0}C, formamide was added to reduce Tm (melting temperature) of nucleic acid hybrids. By including the formamide in hybridization solution, the hybridization temperature can be reduced (Tm-25 ^{0}C) to 30-42 ^{0}C which has several advantages: the probe is more stable at lower temperatures and there is a better retention of noncovalently bound nucleic acids on the membrane. After prehybridization, the solution was removed and replaced by hybridization solution which had the same constituents of the prehybridization mixture except that it contained probe. The purified probe was denatured by boiling it for 10min and was immediately chilled on ice. Hybridization of the probe to the DNA strands on the membrane was continued overnight with gentle shaking. After overnight hybridization, probe was discarded and membranes were washed to remove unbound/unhybridized probe with 2x SSC, 0.1% SDS for 15min twice at room temperature with gentle shaking. Hot wash was given at hybridization temperature with 2x SSC, 0.1% SDS for 10min to 1hr depending on the probe. Washing was continued with stringent conditions with 1x SSC, 0.1% SDS followed by 0.1x SSC, 0.1% SDS depending on autoradiographic signals. Moist membranes were saran wrapped and exposed to X-ray film (Fuji) for 15min to 7 days depending on the signals at -70 ^{0}C using intensifying screens.

Genomic library construction

Although there are quicker methods to isolate genomic sequences corresponding to genes or sequences of interest, the conventional route of screening genomic libraries is a powerful method for isolating genomic sequences of specific interest.

a. Insert DNA preparation: Genomic DNA of Path-6 (7042) was digested extensively with *EcoRI* to obtain mostly the fragment sizes around 4kb as λgt11 can only take inserts upto 7kb size. The genomic DNA fragments generated by *EcoRI* were purified by giving Phenol:Chloroform treatments followed by precipitation with 2.5 volumes of absolute ethanol. The precipitated DNA was washed twice with 70% ethanol, dried and dissolved in sterile water.

b. Ligation of λ arms to digested genomic DNA fragments : *EcoRI* digested λgt11 arms were obtained from Stratagene (Germany). λgt11 is 43 kb in length and can accommodate inserts upto 7.2 kb. Dephosphorylated *EcoRI* digested genomic DNA fragments were ligated to predigested λgt11 arms in a ligation reaction containing 1.0μl (1.0μg) λgt11 arms, 1μl of genomic DNA containing 50-100ng, 0.5μl of 10x ligation buffer, 0.5μl 10mM ATP (pH 7.5), 1μl T_4 DNA ligase (2-3 Weiss units) and 1μl of sterile water to make 5μl final reaction volume. Solution I containing arms, insert and sterile water in an eppendorf vial was mixed with solution II containing ligation buffer, ATP and ligase, and incubated overnight at 4°C. Effectiveness of ligation was checked on 0.6% agarose gel where a positive control with Rheo test insert was used for ligation as suggested by the supplier.

c. Packaging : Packaging extracts are used to package recombinant λphage with high efficiency. We used Giga pack II gold packaging extracts from Stratagene, Germany. Optimal packaging efficiencies were obtained with λ DNAs that were concatameric in nature. Ligations were carried out at DNA concentrations of 200μg/μl which favored concatamers and not circular molecules which only contained one cos site. Gigapack packaging extract is an invitro packaging extract system which preferentially size selects for extra large inserts while maintaining

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highest packaging efficiencies. It also further reduces the background from nonrecombinants.

Invitro packaging was carried out for ligated mix and positive control Rheo test insert and wild λDNA. 1µl of ligated DNA was added to the freeze/thaw extract tube before it began to thaw and to it 15µl of sonic extract was added quickly. It was incubated at 22 ^{0}C for 2hrs after mixing carefully without introducing air bubbles. After incubation, 500µl of phage dilution buffer (SM) [50mM Tris-HCl (pH 7.4), 100mM NaCl, 10mM MgSO$_4$, 0.01% gelatin] was added along with 20µl of chloroform. Lysate was spun briefly to sediment debris and stored at 4^{0}C.

d. Preparation of host bacteria : Single colony from LB plate containing ampicilin of host bacterial strain (Y1090r) supplied by the manufacturer was inoculated onto 5ml LB medium supplemented with 0.2% maltose and 10mM MgSO$_4$ and grown overnight at 37 ^{0}C. Bacteria grown in the presence of maltose adsorb bacteriophage more efficiently. The sugar induces the maltose operon which contains gene (LamB) that codes for the bacteriophage receptor. The overnight grown bacteria were suspended in 5ml of 10mM MgSO$_4$ and cells were finally diluted to 0.5 O.D at 560nm (10^6 cells/ml) with sterile 10mM MgSO$_4$ and kept on ice till further use.

e. Plating : The Y1090r cells in 10mM MgSO$_4$ were used for transduction. The phage lysate was serially diluted in SM buffer in the range of 10^{-2} to 10^{-6} and mixed with above bacterial suspension. The mixture was incubated at 37 ^{0}C for 30min with mild agitation. The bacterial cells with adsorbed phages plated uniformly with molten NZY agarose kept at 45 ^{0}C on NZY agar plates preincubated at 37 ^{0}C and incubated overnight at 37 ^{0}C. Viable phages formed an area of clear (Plaque) zone indicating that phages had multiplied and had infected bacteria. The number of plaques were counted and total plaque forming units (PFU) were determined from the dilution factor. The packaging efficiency was 1×10^7 recombinant plaques ml^{-1}. The recombinant phages obtained on packaging the ligated DNA represented the downy mildew pathogen genomic library.

f. Amplification and storage of the library : The library of recombinant bacteriophages was amplified by growing a plate stock as given above directly from the packaging mixture. Amplified library in the form of lysate was stored at 4 ^{0}C with 0.3% chloroform without reduction in the titer and also in 7% DMSO at -70^{0}C for long term storage.

Screening of genomic library

The original stock of lysate was adsorbed on to fresh host bacterial strain Y1090r as described earlier. The adsorbed bacterial cells were plated on 150mm diametered NZY (Himedia, India) agar media plates with NZY top agarose. The plates were incubated at 37 ^{0}C overnight followed by incubation at 4 ^{0}C for few hours. Plaque lifting was carried out as described by Benton and Davis (174). Nylon membranes were placed on pre-cooled plates containing plaques without introducing air bubbles and were gently lifted and air dried. Membranes were placed in denaturing (0.5M NaOH, 1.5M NaCl) and neutralising (0.1M Tris-HCL pH 7.5, 1.5M NaCl) solutions for 3min each and saturated with 6x SSC. Each membrane was marked for the alignment with the plate. The blots in duplicates from each plate, were air dried, baked at 80 ^{0}C for 3hrs and then stored at room temperature till further use.

a. Plaque hybridization : Prehybridization of plaque lifts was carried out at 65 ^{0}C in hybridization solution for 2-6hrs. 50ng of genomic DNA was radiolabeled by random prime labeling method and was purified on Sephadex column. The prehybridization solution was decanted, fresh hybridization solution was added with radiolabeled DNA and hybridization was continued for 14 -16hrs at 65 ^{0}C. The membranes were washed initially for 15min twice at room temperature with 0.01% SDS and 3x SSC followed by hot wash at 65 ^{0}C with 0.1x SSC and 0.01% SDS. 3-5 rounds of screening were carried out in similar way to isolate single positive plaque.

b. Phage DNA isolation : The overnight grown host bacteria were infected with phage and inoculated in large volume of medium. Initially the concentration of phage was low and uninfected cells in culture continued to divide for several hours. However, successive rounds of infection led to the production of increasing

quantities of bacteriophage. Eventually all of the bacteria became infected and complete lysis of culture occured.

To 100µl of fresh overnight grown bacterial culture approximately 10^6 pfu of bacteriophage were added and incubated at 37 ^{0}C for 20min to allow bacteriophage particles to get adsorbed. Each infected aliquot was added to 100ml of NZY media prewarmed to 37 ^{0}C in 500ml flasks and incubated at 37 ^{0}C with vigorous shaking (250rpm). Concomitant growth of bacteria and bacteriphage occurred resulting in lysis of the culture after 9-12hrs. A fully lysed culture contained considerable amount of bacterial debris. For complete lysis 3ml of chloroform was added and incubation was continued at 37 ^{0}C for 10min more. Then the culture was centrifuged for 10min at 10,000rpm to pellet the cell debris. The supernatant was subjected to ultracentrifugation at 20,000rpm for 30min to pellet the phage particles. The pellet was suspended in 500µl of SM buffer, lysed by treating with 5µl of 20% SDS and 8µl of 0.5M EDTA and incubated at 70 ^{0}C for 20min. This step was followed by phenol:chloroform treatment and precipitation with 3M sodium acetate (pH 5.2) and absolute ethanol overnight. To remove salts, precipitated DNA was treated with 70% ethanol followed by drying under vacuum and dissolving in $T_{10}E_1$. To check the integrity of phage DNA an aliquot was checked on 0.6% agarose gel. Similarly 4µg of phage DNA was digested with *EcoRI* and separated on 0.8% agarose gel to check the insert size

DNA amplification through polymerase chain reaction

The polymerase chain reaction (PCR) is used to amplify a DNA segment that lies between two known sequences (175). Specific primers flanking the target sequence are made to anneal the template DNA followed by extension and denaturation. After 30 cycles, the target sequence is amplified 10^6 fold.

Amplification reactions were performed with 50ng genomic DNA, 200uM dNTPs (New England Biolabs, USA), 40ng of each primer and 1unit Taq DNA polymerase (Bangalore Genei, India) in a 25µl reaction mix using MJR Thermocycler (MJ Scientific, USA), programmed for 40cycles with initial denaturation at 93 ^{0}C for 4min, followed by a temperature regime of 93 ^{0}C for 1min, 50 ^{0}C for 30sec, 72 ^{0}C

for 1min and a final extension at 72 °C for 5min. Amplified products were analyzed by electrophoresis on 1.2% agarose gel in 1x TAE buffer (40mM Tris acetate, 1mM EDTA, pH 8.0) and visualized with ethidium bromide stain under UV light.

Cytofluorometric analysis of sporangial population

Cytofluorometric technique is an appropriate method to study DNA synthetic cycle. This technique involves staining cell suspensions with a fluorescent dye which reacts stoichiometrically with the DNA. The fluorescent pulse amplitude is directly proportional to the amount of fluorescent material present. The amplitude of the pulses resulting from irradiating the cells at a particular wavelength specific to produce fluorescent activation can be used to estimate both, the distribution of cells among phases of the DNA synthetic cycle as well as the total DNA per cell. Measures of relative DNA content reflect ploidy levels. DAPI (4'-6'-diamidino-2-phenylindole) fluorochrome binds primarily to A+T rich DNA molecules (176) whereas propidium iodide intercalates with double stranded nucleic acids and is independent of base composition (177)

Sporangia were treated with absolute ethanol by suspending at room temperature for 10min, followed by 70% ethanol for 30min at 4 °C and resuspended in PBS (Phosphate Buffer Saline). They were treated with RNAse (10µg/ml) for 30min followed by wash with PBS. Spores were incubated with different dyes, (6.9x10^{5}M) propidium iodide, (2.5g/50ml) Hoechst 33256 (178) and (0.1µg/ml in PBS) DAPI (179) (Sigma, USA) for 30min at 4 °C, followed by centrifugation, washing, and resuspension in PBS to a final concentration of 1x10^6 cells/ml. These stained
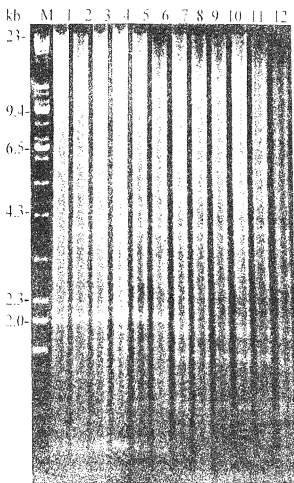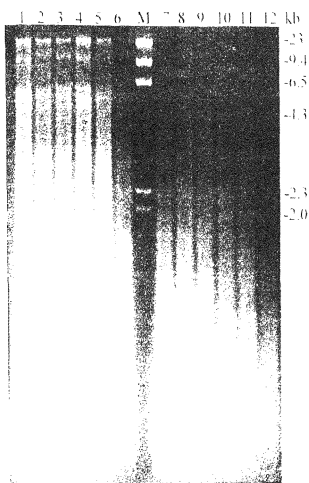
Fig.4a   Fig.4b

Fig.4a :Restriction enzyme digestion pattern of six pathotypes of *S. graminicola*. DNA from six pathotypes were digested with restriction enzymes *Bam*HI (lanes 1-6) and *Hind*III (lanes 7-12), and electrophoresed on agarose gel. Lanes 1&7:path-1 (NHB 3), lanes 2&8:Path-2 (BJ-104), lanes 3&9:Path-3 (MBH 110), lanes 4&10:Path-4 (852B), lanes 5&11:Path-5 (700651), and lanes 6&12:Path-6 (7042S). Lane M:molecular weight marker λ *Hind*III digest and φx174 *Hae*III digest.

Fig.4b :Restriction enzyme digestion pattern of six pathotypes of *S. graminicola*. DNA from six pathotypes were digested with restriction enzymes *Msp*I (lanes 1-6), and *Taq*I (lanes 7-12), and electrophoresed on agarose gel. Lanes 1&7:path-1 (NHB 3), lanes 2&8:path-2 (BJ-104), lanes 3&9:path-3 (MBH 110), lanes 4&10:path-4 (852B), lanes 5&11:path-5 (700651), and lanes 6&12:path-6 (7042S). Lane M: molecular weight marker λ *Hind*III digest.

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sporangia were analyzed in Fac Star plus (Beton & Dekinson) for measuring ploidy level.

Results and Discussion

Restriction enzyme analysis

DNAs of six fungal isolates were digested with a set of restriction enzymes and electrophoresed on agarose gel, revealed a large number of bands on a background smear on ethidium bromide staining. *BamHI* and *HindIII* gave some prominent bands in the high molecular weight range of 2kb and 23kb (Fig.4a). This pattern was more prominent with tetracutter enzymes, such as *TaqI*, and *MspI* (Fig.4b) in the range of 4kb to 500bp than with hexacutter enzymes. These bands are common in all six pathotypes in both the cases. Presence of such bands on restriction enzyme digestion and on agarose gel electrophoresis indicates the presence of repeat elements in *S. graminicola* in an organized pattern.

Identification of repetitive elements

Total repetitive DNA content in *S. graminicola* path-6 was about 8% of which 2.0% was highly repetitive and 6.0% moderately repetitive. This estimate of repetitive DNA is in agreement with those observed in other oomycete obligate fungi. Moderate levels (8%) of repetitive DNA was found in rice blast pathogen, *Magnoporthe grisea* (65). The plant pathogens *Septoria tritici* and *Phytophthora infestans* have much higher levels of repetitive DNA, 26% and 29%, respectively (180; 181). *Neurospora crassa* (182) and *Aspergillus nidulans* have only 1% repetitive DNA consisting of non-ribosomal repetitive DNA which is very low in eukaryotic genomes (183).

Among the highly repetitive clones, eight clones that gave very intense signals were selected from the genomic library for characterization. These clones were further purified to homogeneity through three cycles of plaque hybridization, and hybridized with genomic DNA digested with a selection of restriction enzymes (*BamHI, HindIII, EcoRV,* and *SalI*). All eight repeat elements showed a number of
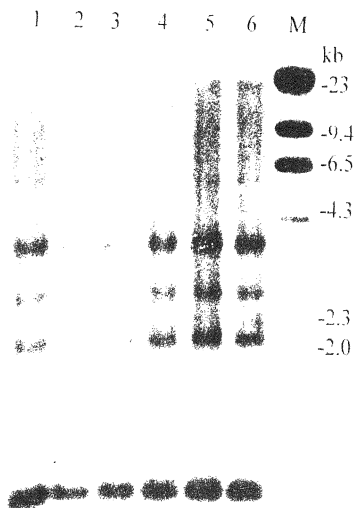
Fig.5

Fig.5 : Autoradiogram of 500bp genomic repeat hybridized with that of *BamHI* digested DNA of six pathotypes of *S. graminicola*: lane 1:path-1 (NHB3), lane 2:path-2 (BJ-104), lane 3:path-3 (MBH-110), lane 4:path-4 (852B), lane 5:path-5 (700651), and lane 6:path-6 (7042S) and lane M λ *HindIII* digested molecular weight marker.
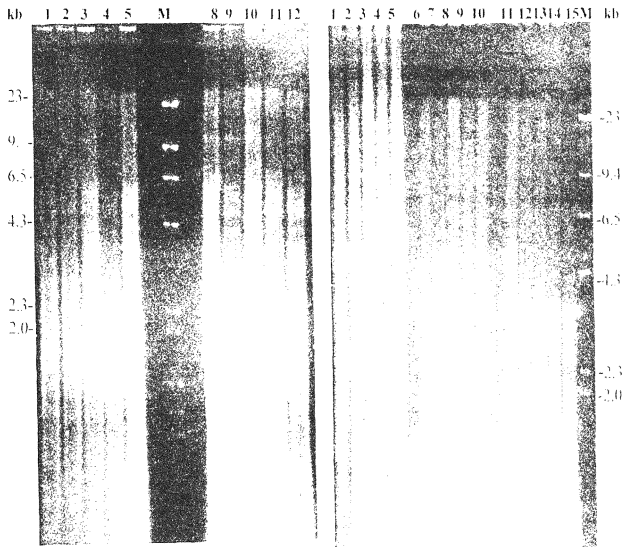
Fig.6a   Fig.6b

Fig.6a :Restriction digestion pattern of five pathotypes obtained with methylation sesitive enzymes *Hpa*II (lanes 1-5) and *Msp*I (lanes 6-10). Lanes 1&6:path-1 (NHB-3): lanes 2&7:path2 (BJ-104). lanes 3&8:path-3 (MBH-110). lanes 4&9:path-4 (852B). and lanes 5&10:path-6 (7042S) and the lane M λ *Hind*III digested molecular weight marker.

Fig.6b :Restriction digestion pattern of five pathotypes with methylation sensitive enzymes *Dpn*I (lanes 1-5). *Sau*3AI (lanes 6-10) and *Mbo*I (lanes 11-15). Lanes 1,6&11:path-1 (NHB-3): lanes 2,7&12:path-2 (BJ-104). lanes 3,8&13:path-3 (MBH-110). lanes 4,9&14:path-4 (852B). and lanes 5,10&15:path-6 (7042S) and the lane M λ *Hind*III digested molecular weight marker.
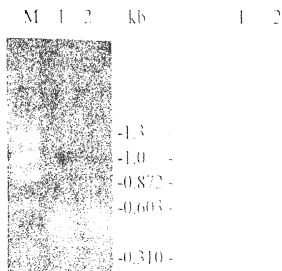
Fig.7a.   Fig.7b
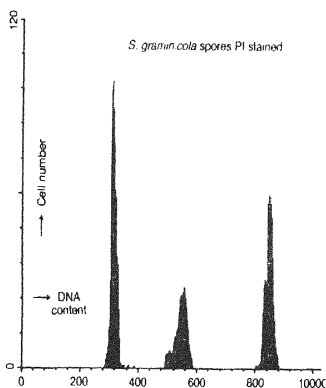
Fig.8

Fig.7a :PCR amplified products of Path-1(NHB3) (lane2) and Path-6(7042S) (lane3) using primers designed to amplify the part of reverse transcriptase gene. Lane 1 is φx174 *Hae*III digested molecular weight marker.

Fig.7b :Autoradiogram showing the hybridization of P5 probe with that of PCR amplified products separated on agarose gel and blotted on nylon membrane. Lane1:Path-1(NHB3) & lane2:Path-6(7042S). Molecular weight marker is on left margin.

Fig.8 :Cytofluorometric analysis of propidium iodide stained sporangia. The X-axis represents DNA content and Y-axis represents sporangial number.

bands on Southern analysis (Fig.5). Multiple hybridizing bands of various molecular weights with all eight repeat elements indicated that a few copies of each repeat element were either clustered in tandem arrays or dispersed in the genome. Hybridization profile with one of these clones λ DM3 with a 500bp insert has been represented in figure 5. Further more we also demonstrated in chapter-III that the presence of microsatellites especially (GATA)$_n$ and minisatellitees (pV47 and R18 like elements) in the genome of *S. graminicola*, confirming the clustered and dispersed arrangement of certain highly repetitive elements.

Methylation

Cytosine methylation at a sequence 5'-- "CCGG"--3' was revealed by the digestion of DNA with isoschizomeric methylation sensitive restriction enzymes *Hpa*II and *Msp*I (Fig.6a). Although there was no difference between overall digestion patterns of both the enzymes, there were some differences in the repeat elements, indicating that the CpC and CpG dinucleotide methylation is prominent in *S. graminicola* repeat elements. Another isoschizomeric adenine methylation-sensitive enzyme set, namely *Sau*3AI, *Dpn*I, and *Mbo*I which recognizes 5'--"GATC"--3' sequence, showed that the digestion by *Dpn*I was less extensive than *Mbo*I and *Sau*3AI revealing partial adenine methylation (Fig.6b). This data reveal that adenine nucleotide in the 5'--GATC--3' sequence of the genome is partially methylated, and the band patterns observed in all the five isolates with *Mbo*I and *Sau*3AI were, identical indicating that there is no cytosine methylation in the sequence of 5'-- GATC--3'. However, the specific variation in the electrophoretic pattern of repeat elements in the genomes digested with methylation sensitive enzymes indicated the predominance of not only `A' methylation in 'GATC' but also CpG and CpC methylation in `CCGG' containing repeat sequences in *S. graminicola* (Fig 6a & 6b).

It is known that actively transcribed genes are under-methylated, and noncoding sequences are highly methylated. DNA methylation plays a significant role in the regulation of gene expression (184).

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Retrotransposable elements

Based on the conserved sequences identified by Xiong & Eickbush (185) in box 1 and 5 of reverse transcriptase sequence of retrotransposons, Weichman & Bussche (75) designed primers to amplify about 343-396bp sequences of the reverse transcriptase gene in all eukaryotic systems. The same set of primers was used to amplify part of the reverse transcriptase gene from the genome of Path-6. A prominent band of 400bp was observed in a PCR reaction (Fig.7a) that was consistent even with altered annealing conditions in PCR. The P5 probe (186) which represents the conserved region of the reverse transcriptase of *Fulvia fulva* was used to hybridize to the PCR-amplified fragment (Fig.7b). PCR amplified fragments showed hybridization with the P5 probe suggesting the presence of retrotransposon-like elements in this fungal genome (Fig.7b). Retrotransposons, the mobile genetic elements, play an important role in genome evolution and constitute about 5-10% of the genome in eukaryotes (187). These elements will be helpful in strain/race identification in *S. graminicola* as shown in yeast (188).

Cytofluorometric analysis of sporangia

Sporangial population was analysed with cytofluorometry using fluorescent dyes such as propidium iodide, Hoechst 33256, and DAPI. Figure 8 shows propidium iodide stained *S. graminicola* of Path-6 spores, revealing the differences in ploidy levels in sporangial population. Cytological studies which yield direct chromosome counts are desirable to confirm our results. Polyploidy, a feature associated with speciation in higher plants was observed in *S. graminicola*. Much need to be learnt about polyploidy and its role in speciation. It is possible that these ploidy differences could be important in establishing genetic diversity in the pathogen population.

In summary, it is for the first time that the genome of *S. graminicola* has been characterized for its repetitive DNA content, methylation status and presence of retrotransposable elements. This study will pave the way to undertake the host-pathogen interaction studies in this system.

CHAPTER-III

GENETIC VARIABILITY ANALYSIS IN
S. GRAMINICOLA USING DNA MARKERS

Introduction

Reasons for studying variation of a specific virulence in plant pathogens relate primarily to the breeding and exploitation of resistant cultivars. While selecting breeding material, it is important to know which pathotype to use in screening process, how resistance is expressed and inherited, and whether it is likely to prove adequate and durable. It is therefore, necessary to study variation in the pathogen population to utilize resistance genes of plants in breeding programs. In order to be efficient in exploiting the host genotypes produced during breeding programs, quantitative data are required on the frequency of occurrence of specific virulence factors and their combination within pathogen population in the geographical area where such resistance is to be employed. A prerequisite for breeding for disease resistance is a knowledge of variation in the pathogen and relation of variation in their aggressiveness and pathotypes.

S. graminicola is heterothallic (39) and the existence of variable pathotypes is known (189). To develop breeding strategies for pearl millet cultivars with stable and durable host resistance to downy mildew, it is important to generate information about the pathogen populations and characterize them at the molecular level.

DNA fingerprinting makes use of minisatellites and microsatellites which constitute part of repeat sequences in eukaryotic genome. Minisatellites or VNTRs (variable number of tandem repeats) comprise 10-40bp long core sequences repeated in tandem and dispersed in the genome of most organisms (190; 191). When these core sequences are hybridized to genomic DNAs digested with restriction enzymes which cut outside the repeat unit leaving the internal repeats intact at relatively low stringency, several hypervariable loci are detected simultaneously (191; 192). Short motifs of 2-10bp long core sequences repeated in tandem and dispersed in the genome constitute simple repetitive DNA sequences (microsatellites), which are observed as major source of genetic variation (193; 194)These simple sequence repeats (SSR) or microsatellites such as (TG)n, (CAC)n, and (GATA)n. are found to be abundant and highly polymorphic in nature. The restriction fragment length

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depends on the number of repeats and thus polymorphism generated between two individuals/pathotypes depends on oligonucleotide probe-enzyme combination used for hybridization (195; 98).

In the present work, the potential of several approaches was examined including a reliable and versatile technique of DNA fingerprinting to study the extent of genetic variation in the pearl millet downy mildew pathogen, *S. graminicola*.

Materials and Methods

Fungal material

Host specific pathotypes : The six host-specific pathotypes of *S. graminicola* used were: Path-1 (from host NHB 3), Path-2 (BJ 104), Path-3 (MBH 110), Path-4 (852 B), Path-5 (700651) and Path-6 (7042S) (150). These pathotypes were maintained on their respective host genotypes by repeated inoculation with asexual spores in isolation chambers in a greenhouse.

Oospore and zoospore isolates : The isolates were collected from different pearl millet growing areas of the Indian sub-continent (50). The sexual spores (oospores) are formed in the leaves infected with thalli of compatible mating types. Such oospore isolates were collected from Coimbatore (designated as CB OS1), Mysore (MYS OS1), Hissar (HS OS6), and ICRISAT (IC OS1). The asexual single zoospore isolates were obtained from sporangia produced on seedlings inoculated with the above oospore isolates. Individual zoospores released from germinating sporangia on 0.75 % water agar were used to inoculate seedlings in pots to induce infection. PT2 and PT3 are opposite mating type isolates and were maintained asexually on highly susceptible pearl millet genotype 7042S.

DNA isolation, restriction digestion and agarose gel electrophoresis

Sporangia were harvested from host specific pathotype and oospore and zoospore infected pearl millet plants. High molecular weight DNA was isolated from sporangia as described in the materials and methods of chapter-II. About 8-10µg of

DNA was digested overnight with 30-70U of the restriction enzymes (according to supplier's instructions) such as *EcoRV, HaeIII, HinfI, HindIII, MspI, PstI, PvuII*, and *TaqI*. DNA digested with hexacutter restriction enzymes such as *EcoRV, HindIII, PstI* and *PvuII* was electrophoresed on 0.8% agarose gels in 1x TPE buffer overnight and stained in ethidium bromide solution and photographed. The gel was destained and used for Southern blotting as described in materials and methods of chapter-II. DNA digested with tetracutter restriction enzymes such as *HaeIII HinfI, MspI &* *TaqI* was electrophoresed on 1.2% agarose gels. These gels were dried and used for Southern hybridization.

In-gel hybridization with end labeled oligonucleotide

a. Synthesis and purification of oligonucleotides : The specific oligonucleotides which were used for hybridization were synthesized on Gene Assembler Plus (Pharmacia) and desalted on a NAP-5 (Sephadex) column. An aliquot of the oligonucleotide was 5' end` labeled with (γ-^{32}P)ATP and checked on a 20% denaturing polyacrylamide gel. If bands lower than desired length of the oligomer were observed, the major band was purified by polyacrylamide gel electrophoresis as follows. 50ml of 20% polyacrylamide gel solution was made with 25ml of 40% acrylamide solution (38:2 acrylamide/bis acrylamide), 5ml of 10x TBE, 21g urea, 80mg ammonium persulphate and 30μl of TEMED. The polyacrylamide gel solution was poured into gel casting unit after sealing the precasted glass plates with molten 1% agarose. 1mm comb was inserted and the gel was polymerized for 45min. Wells were washed with 1x TBE buffer after removal of the comb. The gel was run for 15-30min at 290V in 1x TBE. Equal volume of formamide and xylene cyanol/bromophenol blue mixture was added to the oligonucleotide and heated to 55 °C for 5min to disrupt the secondary structures before loading the samples. The gel was run for 3hrs or until the bromophenol blue reached one end and was stained with ethidium bromide (0.5mg/ml). Using the transilluminator, DNA band of interest was cut into many fine pieces in a 1.5ml eppendorf tube. 500μl of $T_{10}E_1$ was added and incubated overnight at 37 °C. The tube was then spun at 10,000rpm for 10min

and the supernatant containing the purified oligonucleotide was taken out in a fresh tube for further analysis.

b. End labeling : Before using for hybridization, the synthetic oligonucleotides were 5' end labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase catalysing transfer of terminal γ phosphate of ATP to the 5' hydroxyl termini of DNA. 20 moles of oligonucleotide, 5µl of 10x kinase buffer (670mM Tris-HCl, pH 8.0, 100mM MgCl2, 100mM DTT), 5µl of [γ-^{32}P]-ATP, 5U of T4 polynucleotide kinase (Bangalore Genei, India) and sterile water were added to make up volume to 50µl and incubated at 37 ^{0}C for 45min. End labeling reaction was terminated by adding 5µl of 0.2M EDTA. The labeled oligonucleotide was separated from [γ-^{32}P]-ATP by ion exchange chromatography on DE-52 column. Labeled oligonucleotide probe was loaded on 200µl of DE-52 column packed in a 1.5ml eppendorf tube. The column was washed with 4ml of 0.2N NaCl in $T_{10}E_1$. 5' end labeled oligonucleotide was finally eluted by two washes of 500µl each of 1N NaCl in $T_{10}E_1$ and stored at -20 ^{0}C till use.

c. Drying, denaturation and neutralization of agarose gels : For direct in gel hybridization, the agarose gel after electrophoresis was placed on two sheets of thick filter paper and covered with saran wrap. Using a vacuum gel dryer, the gel was dried without heat for an hour or until the gel became completely flat. The gel was heated at 70 ^{0}C for an hour or till it was completely dry and was stored at room temperature till further use. Dried gel was soaked in distilled water until the saran wrap and filter paper were completely separated. It was denatured in 0.5M NaOH/0.15M NaCl for 30min at room temperature and neutralized with 0.5M Tris-HCl pH 8.0 and 0.15M NaCl for 30min at room temperature.

d. Hybridization of dry gels : In dry gel hybridization, neutralized gel was equilibrated in 5x SSPE for 5min and no prehybridization was required. Hybridization was carried out in heat sealed plastic bag containing 5x SSPE, 5x Denhardt's solution, 0.2x BLOTTO and 5'end labeled probe at Tm-5 ^{0}C. Tm was

calculated according to the formula Tm =4 °C (number of G or C) +2 °C (number of A or T). Dry gels were hybridized with oligos at their Tm -5 °C *i.e.,* 35 °C for (GATA)$_4$, 43 °C for (GGAT)$_4$ and (GACA)$_4$ 45 °C for (CAC)$_5$, and 55 °C for (TG)$_{10}$ in hybridization oven for overnight. After overnight hybridization, gels were initially washed thrice for 30min at room temperature with 5x SSC/0.1% SDS and for 2-5min at hybridization temperature with 3x SSC/0.1% SDS. The gel was transferred to a filter paper, dried to remove excess liquid, covered with Saran wrap. Based on the signal further stringent washes were given and exposed to X-ray films (Kodak, USA) at -70°C for a specific period. Dry gels were reused after stripping of the probe by treating with denaturation solution (0.5M NaOH, 0.15M NaCl) followed by neutralization solution (0.5M Tris-HCl pH 8.0, 0.15M NaCl) for 30min.

DNA probes, multiprime labeling and Southern hybridization

A detailed procedure of multiprime labeling and southern hybridization has already been described in materials and methods of chapter II. The repeat sequences were obtained from genomic library of *S. graminicola* by digesting λgt11 clones with *Eco*RI restriction enzyme. The human minisatellite probe, pV47 isolated from human chromosome-6 specific library, was cloned in pUC18 and propagated in JM 101. A 282bp fragment containing nine tandem repeats of 15bp core sequence from M13mp 18RF was obtained by digesting it with *Hae*III and *Cla* I. These probes were labeled with [α-^{32}P]-dCTP by the multiprime labeling method and were used for hybridization overnight in 30% formamide, 5x SSPE, 0.1% SDS, 5x Denhardts, 0.1x BLOTTO at 42 °C with the blots that were obtained by transferring *Eco*RV, *Hind*III, *Pst*I & *Pvu*II digested genomic DNA. The filters were washed twice in 1x SSPE, 0.1% SDS for 15min at room temperature and at 55 °C for 10min for M13, and pV47 as described above except that the hot wash was given at 60 °C for 15min.

MP-PCR fingerprinting

DNA amplification reactions were performed with 50ng genomic DNA, 200ng of dNTPs (NEB, USA) with the addition of 0.15 µCi of [α-^{32}P]-dCTP, 20ng of primer (GATA)$_4$ and 1 unit of Taq polymerase (Bangalore Genei, India) in 25µl reaction mix using MJR Thermocycler (MJ Scientific, USA), programmed for 35 cycles with a temperature regime of 93 °C for 1min, 35 °C for 30sec, 72 °C for 1min and a final extension at 72 °C for 5min. Amplified products were analyzed using electrophoresis on nondenaturing 8% polyacrylamide gels in 1x TAE buffer (40mM Tris acetate, 1mM EDTA, pH 8.0) and detected by overnight exposure to X-ray film (Kodak, USA).

Statistical analysis

The relatedness of the six pathotypes was estimated by means of scorable bands from the various probe-enzyme combinations. Differences in banding pattern were scored on the basis of absence or presence of a band. Similarity indices (D) expressing the probability that a fragment in one pathotype is also found in another for all pair wise comparisons were calculated (X_D represents average similarity index for all pair wise comparisons) (196). Probability that the DNA fingerprints of two pathotypes will be identical by chance was then estimated as (X_D^n). Cluster analysis of data was carried out using the statistical software package SYSTAT 5.1. A dendrogram showing the mean similarities between groups of different pathotypes was generated.

$$\text{Similarity index was calculated as } X_D = \frac{2N_A}{(N_A + N_B)}$$

Where N_{AB} is the number of bands present in both lanes.
N_A is the total number of bands in lane A and N_B is the total number of bands in lane B.

1 2 3 4 5 6 kb

-3.0

Fig.9

Msp-I   Taq-I
1 2 3 4 5 6   1 2 3 4 5 6   kb

-2.0

-1.6

-1.0
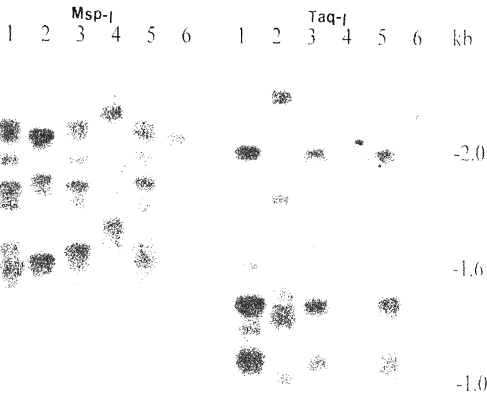
Fig.10

Fig.9 :Autoradiogram showing hybridization of Pv47 minisatellite probe with *PvuII*
digested DNA of six host specific pathotypes of *S. graminicola*. Lane 1 :Path-
2(BJ104); lane 2:Path-3(MBH110); lane 3:Path-1(NHB3); lane 4:Path-4(852B);
lane 5:Path-6(7042S); and lane 6:Path-5(700651). Molecular size markers in kb
are indicated in the right margin.

Fig.10 :Autoradiogram of DNA fingerprints of six host genotype-specific pathotypes of *S.
graminicola*. Fungal DNAs were digested with *Msp*I (lanes 1-6) and *Taq*I (lanes
7-12) and hybridized with (GATA)₄. Lanes 1&7:Path-2 (BJ 104), lane 2&8:Path-3
(MBH 110), lane-3&9:Path-1 (NHB3), lane 4&10:Path-4(852B), lane5&11:Path-
6(7042S), and lane6&12:Path-5(700651). Molecular size markers in kb are
indicated in the right margin.
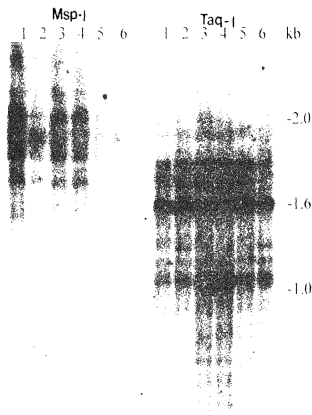
Fig.11
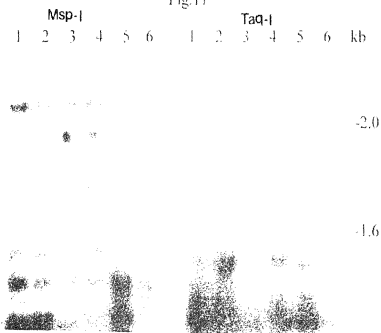
Fig.12

Fig.11 :Autoradiogram of DNA fingerprints of six host genotype-specific pathotypes. Fungal DNAs were digested with *Msp*I (lanes 1-6) and *Taq*I (lanes 7-12) and hybridized with (GACA)₄. Lanes 1&7:Path-2 (BJ 104), lane 2&8:Path-3 (MBH 110), lane-3&9:Path-1 (NHB3), lane 4&10:Path-4(852B), lane5&11:Path-6(7042S), and lane6&12:Path-5(700651). Molecular size markers in kb are indicated on the right margin.

Fig.12 :Autoradiogram of DNA fingerprints of six host genotype-specific pathotypes. Fungal DNAs were digested with *Msp*I (lanes 1-6) and *Taq*I (lanes 7-12) and hybridized with (GAA)₆. Lanes 1&7:Path-2 (BJ 104), lane 2&8:Path-3 (MBH 110), lane-3&9:Path-1 (NHB3), lane 4&10:Path-4(852B), lane5&11:Path-6(7042S), and lane6&12:Path-5(700651). Molecular size markers in kb are indicated on the right margin.
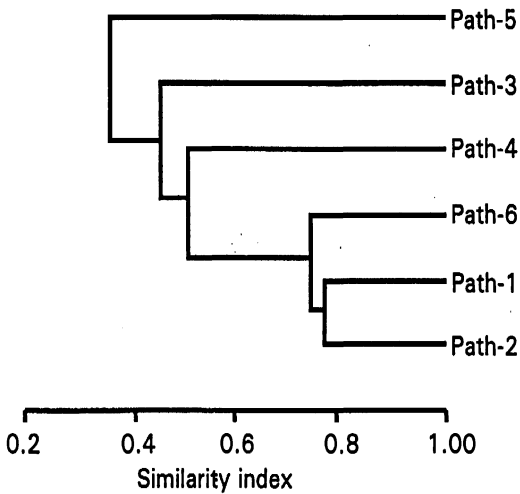
Fig.13

Fig.13 :Dendrogram of six host genotype-specific pathotypes with microsatellite probes.
Cluster analysis based on the data from hybridization of *MspI* and *TaqI* digested
fungal DNA with the microsatellite probes, (GATA)4, (GACA)4, and (GAA)6.
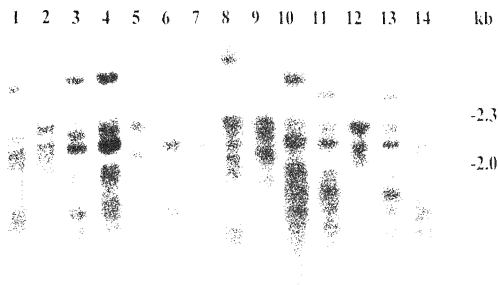
Fig. 14
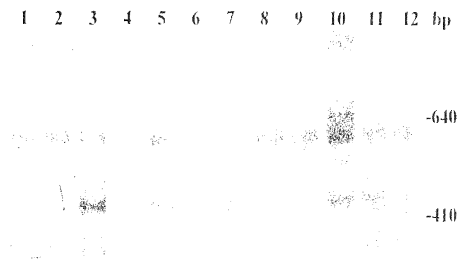
Fig. 15

Fig.14 :Autoradiogram of DNA fingerprints of oospore-and zoospore-derived isolates of
S. graminicola. DNAs of the oospore-and zoospore-derived isolates were
digested with *MspI* and hybridized to the microsatellite probe (GATA)₄. Lanes
1:HS0S6; lane 2:IC0S5; lane 3:CB0S4; lane4:CB0SI Z-1; lane 5:CB0SI Z-3;
lane 6:CB0SI Z-4; lane 7:JPR Z-11; lane 8:PT2; lane 9:PT3; lane 10:PT2 Z-1;
lane 11:PT3 Z-2; lane 12:MYS OSI; lane 13:MYS Z-1; and lane 14:MYS Z-4.
Molecular size of markers in kb are indicated on the right margin.

Fig.15 :Autoradiogram showing the amplification patterns of different isolates of *S.
graminicola* using (GATA)₄ as primer. Lane 1:Path-1(NHB3); lane 2:Path-
3(MBH10); lane 3:Path-4(852B); lane 4:Path-5(700651); lane 5:HS0S6; lane
6:IC0S5; lane 7:CB0S; lane 8:CB0SI Z-1; lane 9:CB0SI Z-3; lane 10:CB0SI Z-
4; lane 11:JPR Z-11; and lane 12:PT2. Molecular size of markers in kb are
indicated on the right margin.

Results

To study variation in six host specific pathotypes of *S.graminicola*, various multilocus probes were used which might reveal polymorphism at many loci simultaneously.

Repeat DNA as markers

Repetitive DNA sequences from a genomic library of the most virulent strain Path-6 constructed in lambda gt11 vector were selected on the basis of strong intensity of hybridization signal with total DNA as detailed in the chapter-II. They were used to detect polymorphism among the six host genotype-specific pathotypes. These probes showed a back ground smear with 5-6 strong bands that were monomorphic.

Minisatellites as markers

The M13 minisatellite probe, known to be useful in detecting high level of polymorphism in human and animals (197), did not show any polymorphism among the six pathotypes. On the other hand, the human minisatellite probe, pV47, known to be very effective in detecting genetic polymorphisms in human and plants (198; 199) was found to be useful in detecting polymorphism among the DNA digests of the six pathotypes of *S. graminicola* with *Pvu*II and this proved to be more informative than other restriction enzyme digests. Path-4 was clearly distinguishable from the other five pathotypes based on a prominent band of 3kb, suggesting that this locus was present only in Path-4 (Fig 9).

Microsatellites as markers

The simple repetitive DNA sequences such as $(GATA)_4$, $(GACA)_4$, $(GAA)_6$, $(CAC)_5$, $(GGAT)_4$ and $(TG)_{10}$ were tested for their ability to detect polymorphism in *S. graminicola* pathotypes using several restriction enzymes. Microsatellites $(TG)_{10}$, $(GGAT)_4$ and $(CAC)_5$ largely produced a smear containing bands that were mostly

monomorphic. Different levels of polymorphisms and complexities with probes (GATA)₄, (GACA)₄ and (GAA)₆ were obtained. Highly polymorphic hybridization patterns unique for each pathotype were observed with (GATA)₄ microsatellite with *Msp*I (lanes 1-6) and *Taq* I (lanes 1-6) enzymes (Fig.10). Five to eight distinct polymorphic bands of 1-3kb size were observed for each pathotype. Path-3, Path-4 and Path-5 (lanes 2,4 and 6, respectively with *Msp*I and lanes 2,4 and 6, respectively with *Taq*I) showed diverse fingerprint patterns with both the enzymes. Path-1 and Path-2 (lanes 1 and 3) showed similar hybridization patterns in *Msp*I digested DNA whereas they could be distinguished from each other using *Taq*I (lanes 1 and 3). Probes (GACA)₄ and (GAA)₆ could also distinguish Path-1, Path-3, and Path-4 from the others (Fig.11 & 12). The average similarity indices among the pathotypes with the probes (GATA)₄, (GACA)₄, and (GAA)₆ were estimated to be 0.26, 0.71 and 0.74, respectively. This clearly shows the hypervariable nature of probe (GATA)₄ and its ability to detect highly polymorphic regions in the genome. Further, to study the stability of the (GATA)₄ derived DNA fingerprints, the pathotypes were examined after 10 asexual generations and were found to be identical.

Table 4: Similarity matrix of six pathotypes with 2 enzymes and 3 probe combinations.

	Path-1	Path-2	Path-3	Path-4	Path-5	Path-6
Path-1	1					
Path-2	0.10	1				
Path-3	0.79	0.07	1			
Path-4	0.23	0.14	0.22	1		
Path-5	0.71	0.26	0.67	0.36	1	
Path-6	0.08	0.14	0.30	0.30	0.21	1

Cluster analysis of pathotypes

A dendrogram based on the DNA fingerprints and similarity index values (table4) obtained with all three probes (GATA)₄, (GACA)₄ and (GAA)₆ clustered the six pathotypes into five groups (Fig.13). Path-5, Path-3 and Path-4 formed distinct

groups, whereas Path-1, Path-2 and Path-6 together formed a separate group in which Path-1 and Path-2 clustered as a subgroup.

Variability study in the oospore and zoospore derived isolates

Since $(GATA)_4$ probe could decipher DNA fingerprints with host-specific pathotypes, we used this probe further to analyze the oospore and zoospore derived isolates. Figure 14 shows *Msp*I digested DNAs from oospore and zoospore isolates hybridized with $(GATA)_4$. Isolates, HS OS6, IC OS5, CB OS4, JPR Z-11, PT2, PT3 and MYS OS1 isolates (lanes 1, 2, 3, 7, 8, 9, and 12, respectively) showed diverse DNA fingerprint patterns which were unique compared to the six host-specific pathotypes. Further, the variation/similarity between individual isolates and different zoospore isolates collected from the same location was also studied. All the three zoospore isolates obtained from Coimbatore showed considerable variation (lanes 4,5 and 6, respectively). On the other hand, the oospore isolate CBOS4 and zoospore isolate CB OS1 Z-1 (lanes 3 and 4, respectively) showed almost similar patterns. Contrary to this, striking differences were observed in the hybridization patterns of oospore and zoospore isolates from Mysore (lanes 13 and 14, respectively). The two opposite mating type isolates PT2 and PT3 (lanes 8 and 9, respectively) showed a high degree of variation. However, the zoospore isolates from Patancheru PT2 Z-1 and PT3 Z-2 showed similar patterns, except for a single band at a molecular weight of 2.5kb (lanes 10 and 11, respectively). Interestingly, PT3 isolate showed almost 100% similarity with Mysore oospore isolate MYS OS1 (lanes 11 and 13, respectively).

DNA amplification fingerprinting

The highly polymorphic nature of oligonucleotide $(GATA)_4$ prompted us to use it as a primer for PCR fingerprinting to amplify the DNAs from the six pathotypes. Most of the bands were monomorphic, and polymorphisms were mainly limited to differences in the intensity of bands (Fig.15). This indicates that there is not much variation in inter SSR sequences and the major variation is due to number of repeats

in various pathotypes. Therefore, we concluded that PCR fingerprinting using (GATA)₄ as primer was not as useful as actual hybridization with simple repeat motifs as reported in yeasts (195).

Discussion

Biological pathotyping indicates existence of variation in the pathogenic populations of *S. graminicola* with reference to the host specificity, aggressiveness, and virulence (50; 200). Pathotyping based on genome similarity adds one more dimension to these studies. Moreover, such pathotyping is more precise and devoid of the influences of environmental factors since the variation is studied at the DNA level. Secondly pathotyping is based on virulence reaction whereas molecular pathotyping is based on general heterozygosity. Genomic repetitive DNA sequences that are believed to be involved in speciation are the main source of polymorphism in eukaryotic system as shown in *Magnaporthe griesa* by Hammer *et al*. (65). In *S. graminicola*, however, repetitive probes from the genomic library were not able to bring out any of polymorphism indicating the highly conserved nature of these repeats in downy mildew fungal isolates.

Variability studies in host genotype-specific pathotypes

Although the human minisatellite, pV47, could distinguish a particular pathotype Path-4, mostly the microsatellite generated DNA fingerprints were more effective in distinguishing all the *S. graminicola* isolates used in the present study. The DNA fingerprint patterns revealed differences in the relative abundance and organization of micro-satellites in the genome of fungal isolates. Polymorphism in the fungal isolates was much pronounced with (GATA)₄, (GACA)₄ and (GAA)₆ which clearly demonstrated the hypervariable nature of simple sequence repeats such as (GATA)₄ that has been well documented in a variety of eukaryotic systems (201; 202). The detection of a high level of polymorphism among the six pathotypes using (GATA)₄

was unique and the other repeat probes described above did not bring about this level of polymorphism with any of the restriction enzymes used in our study. In general, the use of restriction enzymes with four base specificity could decipher more variability in *S. graminicola* than with hexacutter specific enzymes as seen in other systems (203). Microsatellite $(GATA)_4$ derived DNA fingerprints were stable even after 10 asexual generations indicating the utility of this probe in DNA fingerprinting. As shown in our results, the simple sequence repeats/microsatellites were highly informative to develop the pathotype-specific fingerprint patterns. The dendrogram constructed based on fingerprints classified the six pathotypes into five distinct groups. The dendrogram (Fig.13) corroborates well with the characterization of six host genotype-specific pathotypes into five major groups based on differential host reaction (50). The genetic distance among pathotypes can be explained based on their host specificity and genetic dissimilarities among host genotypes. These data agreed well with the groupings based on RAPD patterns of the genotype-specific pathotypes of *S. graminicola* with A-8 primer (204). From the dendrogram, Path-1 (NHB 3) and Path-2 (BJ 104) were considered to be closely related and this could be explained based on the fact that the two host genotypes share a common male parent.

Variability studies in oospore and zoospore derived isolates

We attempted the same enzyme-probe combination mentioned above ($(GATA)_4$ with *Msp*I) to test the usefulness of the probe by studying the different oospore and zoospore derived-isolates. These isolates did not show much homology with the six host genotype specific pathotypes. They also showed much variation within the isolates collected from the same location and a lot of similarities between the isolates from different locations. The variation in the oospore isolates is natural owing to its demographic locations and the adaptation to different pearl millet genotypes growing in that location. The heterogeneity in terms of virulence and aggressiveness was observed among oospore isolates (200). The different zoospore isolates from the same field either represent diverse fungal isolates or they represent a clonal population of the same isolate with variations at the DNA level that could be

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detected with probe (GATA)$_4$. In case of *Septoria tritici*, a wheat pathogen, high level of variability among the isolates was found to be existing in the same lesion or on the same leaf (205). In case of *Magnaporthe grisea*, high degree of genetic diversity was detected within locations in the same field (206). This supports the finding that differences at the DNA level are distributed on a very fine spatial scale in several fungi (207; 208; 206) which can be detected only with the help of a sensitive tool such as DNA fingerprinting. There are reports that oligonucleotide probes can detect variations even in clonal populations (209).The combined influences of the introduction of new host genotypes, step wise selection for virulence and possibility of other mechanisms for genetic reassortment may have contributed to the development of new virulent races. However, at the molecular level, there seems to be a limited variation among the pathotypes and may be confined to a specific locus in the genome that could be detected only with the help of hypervariable probes. DNA fingerprinting demonstrates a very high level of genetic variation in natural populations indicating that these populations probably contain sufficient genetic variability to allow for rapid selection of clones that are resistant to fungicides or virulent on pearl millet varieties having pathotype-specific resistance. These data provide sufficient insight into population structure and clonal dynamics of the downy mildew pathogen.Thus the use of simple sequence repeats such as (GATA)$_4$ which is highly polymorphic has proved to be very useful for rapid and efficient identification and genetic analysis of the pathogen population. These fingerprint profiles of different pathotypes can be used as diagnostic tools to formulate breeding strategies targeting resistance to local population and for monitoring the emergence of new virulent races.

CHAPTER-IV

GENETIC BASIS OF ADAPTABILITY OF *SCLEROSPORA GRAMINICOLA* TO INDIVIDUAL HOST GENOTYPES OF PEARL MILLET

Introduction

S. graminicola is completely host-dependent and by virtue of its systemic growth, can alter host growth and manipulate host reproduction. It is known for its rapid adaptability to new resistant genotypes leading to substantial yield losses. Downy mildew epidemics occur at regular intervals of once every three years on an average and were reported during 1970-76 (33), 1983-84 and again in 1987-88 (35). During these epidemics, several hybrids were withdrawn because of their susceptibility to downy mildew.

In the previous chapters, I have described genome analysis and genetic variability of *S. graminicola* pathogen. In this chapter, the changes in the host-selected pathotypes were examined by inoculating them onto nonspecific host genotypes of pearl millet and maintaining them for 10 asexual generations on the same genotypes. At alternate generations, the pathogenecity of each pathotype was tested on its original host genotype and on the new host genotype. The changes in the pathotypes at the molecular level were also monitored using microsatellites and RAPDs as they adapted to the new host genotypes.

Materials and Methods

Fungal pathotypes and host genotypes

Two host selected pathotypes Path-1 (isolate Sg 008) and Path-5 (Sg 012) and their respective pearl millet host-genotypes NHB 3 and 700651 were selected for this study. These pathotypes were identified from Patancheru population (Sg 001) from a mix of pearl millet genotypes 7042S, and NHB 3 tested on potential host differentials and maintained in polyacrylic isolation chambers in a green house. Seeds of pearl millet genotypes NHB 3 and 700651 were surface-sterilized with 2.5% NaOCl for 5min followed by thorough washing with water and were sown as 20 seeds per pot. Pots for each treatment were kept isolated in polyacrylic boxes to avoid cross contamination among the isolates. Inoculum prepared from leaves previously infected with individual pathotypes as described in chapter II, was inoculated at the first leaf stage on seedlings which were maintained in a greenhouse at 25 ± 2 °C. The seedlings were observed for downy mildew symptoms from the 4th day after inoculation to determine the latent period. The

number of infected seedlings and total seedlings per pot were recorded 10 days after inoculation to determine the percentage of infection.

Swap experimental design

Isolates of Path-1 and Path-5 were swapped by cross-inoculating them on pearl millet genotypes 700651 and NHB 3, respectively and maintained for 10 generations asexually. These generations on new host were designated as AG_{10} (altered host generation 10). To serve as controls, these two pathotypes were maintained on their respective original host genotypes for the same number of generations and were designated as G_{10} (Fig. 16). Pathogenecity was measured as the ability of the pathotype to produce infection (symptoms) in terms of latent period and the percentage of infected seedlings (% incidence) (197). Virulence and latent periods were tested on alternate generations with two replications for each treatment, and with about 40 seedlings per replication.

The treatments were: Path-1 on its original host NHB 3, on its new host 700651, and on NHB 3 isolated from 700651 every alternate generation; similarly, Path-5 on its original host 700651, on its new host NHB 3, and on 700651 isolated from NHB 3 every alternate generation (Fig. 16).

DNA isolation, restriction enzyme digestion and agarose gel electrophoresis

Sporangia were collected from sporulated, infected leaves after overnight incubation at 20 °C. High molecular weight DNA was isolated from sporangia as described in materials and methods of chapter-II. Restriction enzymes *Msp*I and *Taq*I were used individually to digest about 8-10µg of genomic DNA according to supplier's instructions and electrophoresis was carried out on 1.2% agarose gel in TPE buffer. The gels were stained in ethidium bromide, photographed and dried in a gel dryer.

DNA probes and Southern hybridization

Oligonucleotides were synthesized on a gene assembler plus (Pharmacia), desalted on a NAP column and purified on a 20% denaturing polyacrylamide gel as described in meterials and methods of chapter-III. Oligonucleotide probe was 5'-end labeled by T_4 polynucleotide kinase. Dry gels were denatured, neutralized and

hybridized at their Tm-5 °C, i.e. 35 °C for $(GATA)_4$ overnight and subjected to stringent washing conditions. Gels were initially washed thrice for 30min at room temperature with 5x SSC/0.1%SDS and for 2-5min at hybridization temperature with 3x SSC/0.1% SDS. The gels were transferred to a filter paper, dried to remove excess liquid, covered with Saran wrap and exposed at -70 °C. The hybridized gels were exposed to X-ray films for specific periods, depending upon the intensity of the hybridization signal.

Polymerase chain reaction

RAPD reactions were performed in a 25ml volume containing 10 x Taq buffer (Promega), 100mM dNTPs (NEB), 15ng primer (10-mer Operon), 50ng of genomic DNA and one unit Taq DNA polymerase (Promega) essentially as described by Williams *et al.* (175). Amplification was carried out in a MJ Research DNA engine (PTC 200) thermal cycler programmed for 5min at 94 °C followed by 40 cycles of 1min at 94 °C, 1min at 36 °C and 2min at 72 °C. Amplified fragments were resolved by electrophoresis on a 2% agarose gel containing 0.5µg/ml ethidium bromide and the bands were visualized on a UV transilluminator.

Data on latent period and disease incidence were subjected to analysis of variance using GENSTAT (Rothamsted Experiment Station, Harpenden, Herts AL5 2JQ, UK) to determine the significance levels of different treatments. Regression analysis was done to determine the relationship between generation and disease incidence.

Results

We attempted to study the changes in Path-1 and Path-5 after host-pathogen swap experiments in terms of pathogenecity, latent period, and disease incidence by using molecular markers such as RAPDs and DNA fingerprinting.

Host specificity

G10 ◄ – – – Path-1   Path-5 – – – ► G10

(specific to cultivar NHB3)   (specific to cultivar 700651)

cross inoculation

NHB3 (AG1)   700651 (AG1)

(Path-5, G10)   (Path-1, G10)
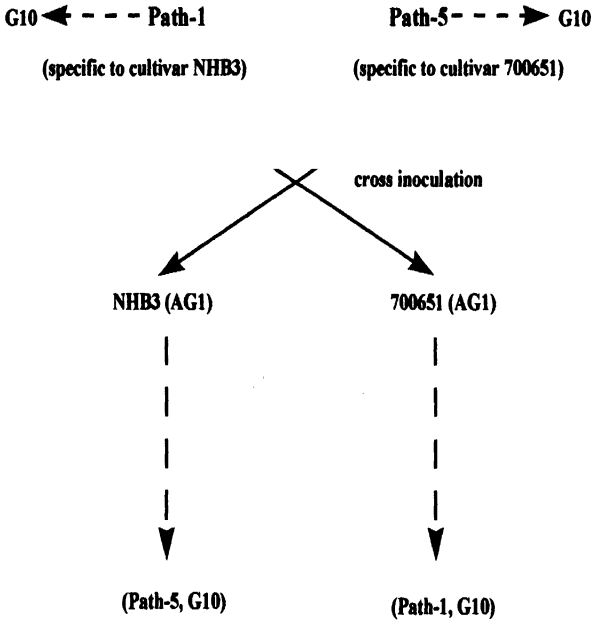
Fig. 16

Fig. 16: Schematic representation of host-pathogen cross-inoculation experiments Path-1 selected on NHB3 and Path-5selected on 700651 were cross-inoculated on to their new hosts and maintained for 10 generations. At every alternate generation, the virulence and latent period were tested on the original host and the new one. Path-1 and Path-5 maintained on their original hosts served as controls.

Disease incidence

Significant increase in disease incidence occurred for Path-1 on the new host 700651 from AG_1 (21%) to AG_{10} (45%) (Table 5), and a significant linear relationship was found between generation and disease incidence (Fig. 20a). However, a similar increase in disease incidence was not observed for Path-5 on new host NHB 3. The disease incidence on NHB 3 was significantly reduced with Path-1 obtained from the cross inoculated genotype 700651 compared with the one maintained on the selected host NHB 3. This decline in disease incidence had a significant negative relationship with increasing asexual generation (57% at G2 to 26% at G10 (Fig.20b). There was no such change in disease incidence for path-5 from its new host NHB 3 to its original host 700651 at different generations of testing. The two controls, Path-1 and Path-5 on their respective hosts showed no significant change for disease incidence across generations. Highly significant effects were observed with treatments, asexual generations and their interaction on disease incidence (Table 5), although the treatment effects were much larger than the other two.

Table 5: Downy mildew incidence (%) caused by host specific pathotypes of *S. graminicola* in a cross-inoculation experiment maintained for 10 asexual generations under glass house conditions

Treatments	DM incidence (%) at asexual generation number				
	2nd	4th	6th	8th	10th
Path-1 on NHB 3	83	76	84	75	77
Path-1 on 700651	21	27	33	35	45
Path-1 (ex 700651) on NHB 3	57	56	38	27	26
Path-5 on 700651	29	39	34	27	32
Path-5 on NHB 3	36	37	37	38	37
Path-5 (ex NHB 3) on 700651	9	10	9	8	9
SE (m)	± 1.9	± 1.47	± 3.4	± 0.7	± 1.5
Mean	39	41	39	35	37

Percentage downy mildew incidence; mean of 2 replications.

Latent period varied significantly at different generations with both pathotypes on

the new host genotypes. For Path-1 on 700651, the latent period reduced from 10 days at generation 2 (AG_2) to 6.5 days at generation 10 (AG_{10}) (Table 6). Similarly, for Path-5 on NHB 3 the latent period reduced from 8 days at AG_2 to 6.5 days at AG_{10}. Similar observations were made for Path-5 from NHB 3 to 700651 at different generations. However, there were no significant differences for Path-1 and Path-5 on their respective hosts genotypes at different generations. Analysis of variance showed significant effects of treatments, asexual generations and their interaction on latent period.

Table 6: Latent period (days) of host-specific pathotypes of *S. graminicola* in a cross-inoculation experiment maintained for 10 generations under glasshouse conditions

Treatments	Latent period (days) at asexual generation number				
	2nd	4th	6th	8th	10th
Path-1 on NHB 3	6.0	5.5	5.5	6.0	5.5
Path-1 on 700651	10.0	8.0	8.0	7.0	6.5
Path-1 (ex 700651) on NHB 3	8.0	8.0	8.0	6.5	6.5
Path-5 on 700651	8.0	7.0	7.5	7.5	7.5
Path-5 on NHB 3	8.0	8.0	7.5	7.0	6.5
Path-5 (ex NHB 3) on 700651	10.0	8.0	8.0	7.0	8.5
SE (m)	± 0.0	± 0.20	± 0.27	± 0.32	± 0.55
Mean	8.3	7.4	7.4	6.8	6.8

Latent period (days); mean of two replications.
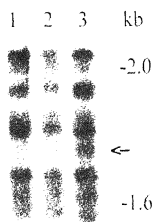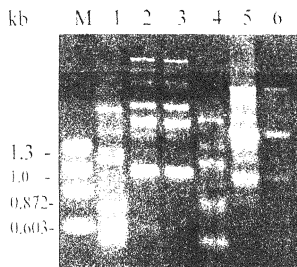
Fig.17   Fig.18
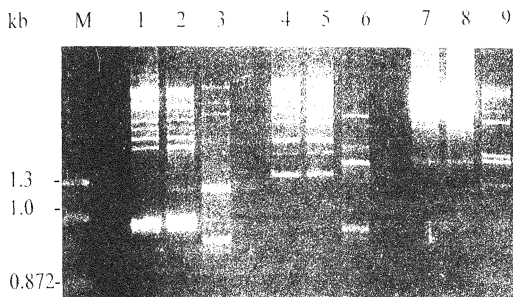
Fig.19

Fig.17 :DNA fingerprinting with different generations of Path-1 with (GATA)₄. Path-1
DNAs was digested with *Msp*I. Lane1:Path-1 on NHB3 at G_{10}; lane 2:Path-1 on
700651 at G_0; and lane 3:Path-1 on 700651 at AG_{10}. A band unique to lane 3
(Path-1 on 700651 at AG_{10}) is indicated by an arrow. Molecular weight marker is
indicated on right margin

Fig 18 :RAPD pattern with different generations of Path-1 using oligonucleotide primers
J9 (lanes 1-3) and J18 (lanes 4-6). Lane 1&4:Path-1 on 700651 at AG_{10}; lanes
2&5:Path-1 on 700651 at G_0, lanes 3&6:Path-1 on NHB3 at G_{10}. Lane M is
ϕx174 *Hae*III digest molecular weight markers.

Fig19 :RAPD pattern with different generations of Path-5 using oligonucleotide primers
I1 (lanes 1-3), I10 (lanes 4-6) and I11 (lanes 7-9). Lanes 1,4& 7:Path-5 on
700651 at G_{10} Lanes 2,5&8: Path-5 on NHB3 at G_0, lanes 3,6 & 9:Path-5 on
NHB3 at AG_{10}. Lane M is ϕx174 *Hae*III digest molecular weight markers.

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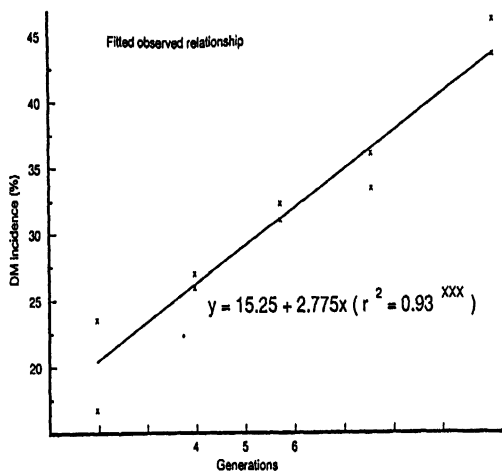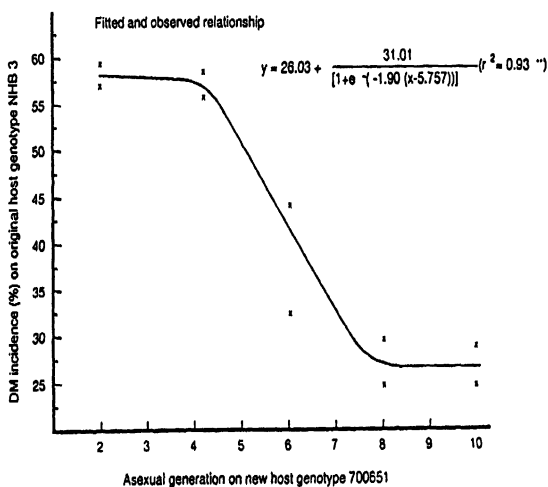
Fig. 20a

Fig.20b

Fig.20a&b:Adaptation of a host selected pathotype (NHB3 Path-1) to a new host
(700651) as indicated by the regression curves of asexual generation and
downy mildew incidence as a function of A=Path-1 on 700651; and
B=Path-1 on NHB3 through 700651. Note that r^2 values are significant in
both the cases.

Identification of DNA polymorphism

As described in chapter III, pathotype specific individual fingerprint patterns were generated using microsatellites for host selected pathotypes. The same enzyme-probe combinations were tested in this study, on Path-1 and Path-5 before and after changing host genotypes. Figure 17 shows the DNA fingerprint pattern of Path-1 using $(GATA)_4$. Lane 2 in this figure shows the fingerprint pattern of Path-1 as control (G_0). Lane 1 is a fingerprint pattern of Path-1 maintained on its original host genotype NHB 3 for 10 generations (G_{10}) and lane 3 is a fingerprint pattern of Path-1 obtained after maintaining 10 generations on its new host 700651 (AG_{10}). A new fingerprint band of size 1.7kb appeared in the AG_{10} (lane 3) that was absent in G_0 (lane 2) and G_{10} (lane 1). This band was in addition to the original fingerprint pattern of Path-1. No such change in the fingerprint pattern of Path-5 was observed before or after, several passages through new host genotype.

The pathotypes were further screened with RAPD primers before infecting the new host genotype (generation G_0) and after maintaining 10 asexual generations on a new host genotype (generation AG_{10}). The control was maintained under the same conditions on its selected host genotype for 10 generations (G_{10}). Path-1 and Path-5 were screened with 120 oligonucleotide (Operon) primers to detect polymorphism. Out of 120 primers, two primers (J9 and J18) could distinguish AG_{10} and G_0 in Path-1 (Fig.18) whereas three primers (I1, I10 & I11,) could identify the differences between generations of Path-5 (Fig.19). Each primer amplified three to four intense bands with each treatment. But mostly the differences were limited to a few bands variation which were limited consistent in each pathotype.

In plants and fungi reports attributing functions to microsatellies are not known so far. In rice (210) and in some other plant species (211), it has been shown that microsatellites are transcriptionally active. Increase in the number of trinucleotide repeats, has been shown to be correlated with some genetic diseases (212) especially in humans. This change in fingerprint pattern may be due to increase in repeat numbers of microsatellites or point mutations in the region of recognition sequence of the restriction enzymes and primer binding sites in the case of RAPD markers. The appearance of a new band in the

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fingerprint pattern (Fig.17) and polymorphism revealed by RAPDs (Fig 18 & 19) need to be further tested to correlate with changes in host specificity and reduced virulence on its original host genotype.

Discussion

Analysis of host-specificity of the pathogen population

Serial advancement of generations in case of Path-1 on its original host genotype NHB 3 did not show much change in the latent period as well as disease incidence. However, when the host genotype was changed to 700651, the latent period was initially much higher which decreased gradually in the tenth generation. On the other hand, the disease incidence on this new host (700651) increased slowly with the generations. In both the cases (latent period and disease incidence), the results indicated a slow adaptation of Path-1 to its new host. Again when the adapted Path-1 was tested on its original host NHB 3, initial generations showed more disease incidence than the later generations. These observation indicate adaptability of Path-1 to new host genotype. Pearl millet genotype NHB 3 grown at Durgapura (Rajasthan, India) in 1977 succumbed to downy mildew. However, the same genotype was unaffected by the disease when grown 3 years later in the same sick plot (213). This observation is in agreement with the hypothesis of slow adaptation of Path-1 to its new host 700651 put forth by us. However, the conclusion drawn by Singh & Singh (213) that a pathogen population specific to a particular genotype can disappear within 3 years, needs to be modified. The pathogen specific to a particular genotype gets adapted slowly to a new genotype and it cannot then efficiently and rapidly revert back to its original host. Similar results were obtained when the same experiment was repeated with the same genotype at Nakshatrawadi (Maharastra, India). It has been observed that there are regular shifts in virulence of pathogen population with changes in host genotypes (213).

In case of Path-5, however the situation is exactly opposite (Table 5 &.6). Path-5 could adapt to the new host NHB 3 very easily as revealed by almost similar latent period and incidence at each generation. This can be explained by the fact that the field isolate was originally maintained on NHB 3 before making the

selection on a specific host genotype. On the other hand, Path-5's reverting back to its original host (700651) was not as efficient although the latent period and disease incidence remained unchanged across generations. Initial adaptability of Path-5 to its new host was more efficient than that of Path-1 although its virulence was lower than that of Path-1. The low virulence of Path-5 on 700651 was also due to heterogeneity in the host population, and the disease incidence was never higher than 40% (R.P. Thakur, ICRISAT, personal communication). The broader host specificity of Path-5 can, therefore, result in a quick break down of downy mildew resistance irrespective of the rotation of pearl millet genotypes, if a previously susceptible cultivar is brought back into cultivation.

The emergence of a new pathotype in an asexual population is not solely an outcome of genetic recombination, but could also be the effect of host genotype directed selection for specific virulence in a variable pathogen population. Specificity can be determined by sequential genetic interactions between plant and pathogen. When a host specific pathogen is used to infect a new host genotype, a whole range of genes that are involved in early recognition events and virulence need to be expressed. Host-race specific recognition may occur by the direct interaction of the "*avr*" gene product with the corresponding plant "R" gene product (103). According to Flor's hypothesis (214), the genotype specificity within a host species is often determined by gene for gene interactions. Newton and Crute (105) put forth the genetic evidence suggesting that gene for gene relationships might also control host species specificity.

We are dealing with a population of pathogen isolates, from which the isolate that is selected against a host becomes dominant over the generations. With the passage of several generations on a specific host genotype, the isolate showing higher virulence increases in proportion to the less virulent ones. The change in host-specificity can be explained either as an adaptation of the most virulent species to the newly selected host genotype involving change in mechanism of host-pathogen interaction, or a selective enrichment of the previously less virulent species from the pathogen population to the new but specific host. The fact that downy mildew is a quantitative trait with many loci having variable levels of resistance also lends support to the view that host adaptation is conditioned by the different *R* genes present in the host (151). To study this

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further we may need single spore isolates that multiply in a specific host.

In summary, understanding the molecular mechanisms that govern host species/cultivar/genotype-race specificity aids in developing new strategies for control of biotic stresses. One of the main reasons for the break down of disease resistance is changes in genotype specificity. Our work throws some light on the concept of genetic adaptation of *S. graminicola* to the individual host genotypes resulting in reduced fitness on the original hosts.

ASSESSMENT OF DOWNY MILDEW INDUCED RESPONSE IN PEARL MILLET USING DDRT-PCR APPROACH

Introduction

The responses of plants to environmental stresses are characterized by complex physiological and biochemical changes that ultimately result from the selective increase or decrease in the biosynthesis of a large number of distinct proteins. These changes in protein patterns are due, at least in part, to changes in the transcriptional activity of the corresponding genes. The initial encounter between a potential pathogen and host plant involves series of events which are preexisting or induced defenses by the host and aggressive or counteractive responses by the pathogen. Such interactions based on the compatibility between host and pathogen determine resistance or susceptibility of the host.

Analysis of differential gene expression relies on hybridization procedures (215) which have been successfully utilized in the analysis of plant-pathogen interaction to isolate infection structure-specific genes (216; 217) and genes specially induced during pathogenesis (218; 219). These procedures are laborious, time-consuming and their sensitivity is limited allowing detection of only relatively abundant mRNAs.

Differentially display of RNA from different tissues has recently been described as a powerful technique for the isolation of tissue-specific or stage-specific genes (220-222). This technique is based on the random amplification of fragments from first strand cDNA using combination of an anchored poly-dT primer, a random decamer primer and is able to amplify from 50 to more than 100 fragments from different genes simultaneously. In fact it has been suggested that the entire mRNA population of a given tissue can be monitored in a relatively short time with a limited number of primers (220).

To analyze host-pathogen interactions and to understand the signaling events that occur between pearl millet and *S. graminicola,* DDRT is the most ideal technique especially where the pathogen cannot complete its life cycle without the host plant. In the present work we report isolation of two differentially expressed mRNAs by the pathogen in infected host plant.

Materials and Methods

Fungal material

Highly virulent pathotype-6 (IC7042) was selected in this study. Sporangia were harvested from sporulating leaves in ice cold sterile distilled water. Inoculum was prepared as described in materials and methods of chapter-II. Sporangia were inoculated on first leaf stage seedlings with a 5µl drop of sporangial suspension (1X10^5 sporangia/ml) and kept overnight at 20 ^{0}C and 95% relative humidity. The infected plants were grown for about 20 days in isolation chambers. Sporangia were harvested from infected leaves as described in chapter-II, and used as source material for fungal DNA and RNA isolation.

Plant material

Pearl millet cultivars7042 - resistant and susceptible to downy mildew were used for this study. Pearl millet seeds were surface sterilized with 2.5% NaOCl for 5min. followed by thorough washing with water and were sown in plastic trays on vermiculite. Seedlings were grown under 25±2 ^{0}C temperature, luminous light conditions for 8hrs and rest in dark in incubators, supplemented with Hogland nutrient solution. Inoculations were carried out on resistant and susceptible cultivar sets and one set of susceptible cultivar was kept as healthy control. Seedlings were collected on the sixth day after inoculation and washed with DEPC treated water and frozen in liquid nitrogen.

Total RNA isolation

A good preparation of RNA is an essential step in gene analysis and gene expression studies. The main problem in RNA isolation is the presence of ribonucleases in all the tissues. Therefore, the various steps involved in RNA isolation include inhibition of ribonucleses, followed by deproteinization and separation of RNA from other components of the homogenate. In the protocol used in our studies deionized water was treated with 0.1% diethylpyrocarbonate (DEPC) and all the glassware was washed with DEPC water, autoclaved and baked at 160 ^{0}C overnight to inhibit RNAse activity. Frozen plant material and fungal sporangia (~1g) were

ground to fine powder in liquid nitrogen. To 1g of powdered material a hot (60 ^{0}C) mixture of 4ml RNA isolation buffer (0.2M sodium acetate pH 5.0, 0.1% SDS, 10mM EDTA) and 4ml distilled phenol were added. The suspension was vortexed for another 10min and centrifuged at 10,000rpm for 10min. The aqueous phase was removed, and re-extracted with chloroform. RNA was precipitated overnight at 4 ^{0}C, in 2M LiCl and pelleted by centrifugation at 10,000rpm for 10min. The RNA pellet was washed once with 2M LiCl and twice with 80% ethanol and dried under vacuum. The pellet was dissolved in sterile water, concentration was estimated by spectrophotometer and stored at -70 ^{0}C. RNA integrity was verified by denaturing formaldehyde agarose gel electrophoresis.

DNA isolations were carried out from fungal sporangia as described in the Materials and Methods of chapter-II.

Northern blotting and hybridization

The size and amount of specific mRNA in preparations of total or poly(A)+ RNA can be determined by northern blotting and hybridization. RNA is separated based on size by electrophoresis through denaturing agarose gel and is then transferred to a nylon membrane. The RNA species of interest can be detected by hybridization with a radio-labeled probe.

Approximately 30μg of total RNA sample was incubated at 65 ^{0}C for 5min with 12.5μl of formamide and chilled on ice. 2.5μl of 50%[v/v] glycerol containing 0.1mg/ml bromophenol blue and xylene cyanol was added and electrophoresed on 1.2% agarose gels containing 7.4% formaldehyde, 1x MOPS buffer, 5mM sodium acetate and 1mM EDTA. After electrophoresis, RNA gel was transferred on to Hybond N membranes (Amersham, UK) by vacuum blotting (LKB Pharmacia) and the RNA was irreversibly bound to the membrane by UV cross linking. 1g agarose was dissolved in sterile water and cooled to 50 ^{0}C. 10ml of 10x MOPS buffer and 17ml formaldehyde were added to agarose solution, mixed and poured immediately. The gel was run at 40mA in 1x MOPS running buffer and stained in ethidium bromide, destained and photographed on transilluminator.

DNAse treatment of total RNA

1µg of total RNA was treated with 20U of RNAse free DNAse (Amersham) for 1hr at 37 ^{0}C along with 10U of RNAsin (Promega) in the reaction mixture. The DNAse was heat denatured at 65 ^{0}C for 5min. and extracted with Phenol:chloroform mix, precipitated, dried and treated with 70% alcohol to remove salts. RNA was again subjected to electrophoresis on denaturing formaldehyde agarose gel to check its integrity.

First strand synthesis

DNAse treated RNA was reverse transcribed by using $T_{12}MA$, T_{12} MC, $T_{12}MG$ and $T_{12}MT$ anchored primers where M stands for dA, dC, dG, and dT (Genhunt, USA). First strand was synthesized using Boehringer Mannheim kit according to the manufacturer's instructions. RNA along with sterile water was denatured at 65 °C for 10min and incubated at 25 °C along with buffer, and primer for 10min. AMV reverse transcriptase (20 units) along with 1mM dNTPs, and 6mM MgCl2 was added and incubated at 42 °C for 60min. Reverse transcriptase was denatured by incubating the sample at 99 °C for 10min.

PCR amplification reactions were performed in 10µl reaction volume containing 1µl of first strand reaction mix, 10µM $T_{12}MN$ primer in combination with 2µM arbitrary 10bp Operon primer, 25µM dNTPS, 1unit Amplitaq (Perkin Elmer) in the presence of 0.1µCi [α-^{32}P]-dCTP. The PCR was programmed for 40 cycles with temperature regime of 30sec at 94 ^{0}C, 2min at 40 ^{0}C, and 30sec at 72 ^{0}C, and with final extension for 5min. at 72 ^{0}C in MJR thermocycler (MJ Scientific, USA)

PCR amplified fragments were separated on denaturing 6% polyacrylamide gels in TBE buffer to size fractionate the amplified cDNA products. The denaturing polyacrylamide gel was run at 2,500 volts, 30mA and 40watts for 4 to 5hrs till the bromophenol blue touched the lower edge of the gel. The gels were exposed to X-ray films (Indu, India) for overnight after marking them for alignment by poking with needle on 4 corners of the film.

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Recovery of bands

Polyacrylamide gel and X-ray film were aligned and bands of interest were cut out from the gel. DNA from gel slices was recovered by using Qiagen gel extraction column (Qiagen, USA). DNA was eluted in 30μl volume and 15μl out of it was used for reamplification using the same primer combinations and PCR conditions except 25μM dNTP concentration. The amplified products were run on a 2% agarose gel and fragments were again purified by Qiagen gel extraction kit columns. These amplified bands were cloned using pMOS T-vector kit (Amersham, UK). More than one white colonies were selected from each plate.

Northern and Southern blot analysis

The cloned bands were used as probes in Northern and Southern blot analysis. Hybridizations were carried out overnight at 60 ^{0}C in 7% SDS, 1% BSA, 0.5M sodium phosphate buffer (pH 7.4) and 1mM EDTA. [α-^{32}P]-probes were generated using random primer labeling kit (BARC, India) (173). Washing steps were performed at room temperature for two times for 15min each with 3x SSC, 0.1% SDS followed by 1x SSC, 0.1% SDS at 60 ^{0}C for 10min. Blots were exposed to X-ray films (Indu, India) overnight to week depending on the intensity of the signal.

DNA sequencing

DNA sequencing as described by Sanger *et al.* was carried out using Sequenase version 2.0 kit (USB, UK) according to manufacturer's instructions. This involves the use of specific primer for extension by a DNA polymerase, chain termination by dideoxynucleotide phosphates and the use of polyacrylamide gels to separate single stranded DNA chains capable of resolving a single nucleotide difference. 2', 3' ddNTPs differ from dNTPs in the absence of hydroxyl residue at 3' position of deoxy ribose which prevents the formation of phosphodiester bond with succeeding dNTPs, resulting in termination of the reaction. A small amount of ddNTP is included with the four conventional dNTPs in a reaction mixture along with DNA polymerase which results in competition between extension of the chain and infrequent but specific termination. By using four different ddNTPs in four separate

reactions, oligonucleotides are generated that terminate at positions occupied by every A, C, G, or T in template strand. The DNA polymerases generally used for sequencing include Klenow fragment of *E. coli* DNA polymerase-1 and Sequenase version 2.0 enzyme (a genetically engineered and modified T7 DNA polymerase to eliminate 3' → 5' exonuclease activity).

a. Denaturation of template DNA : 2μg of plasmid DNA was dried in 1.5ml vial and dissolved in 40μl of denaturation buffer (0.2M NaOH, 0.2mM EDTA, pH. 8.0) and kept at 37 °C for 30min. 4μl of 3M sodium acetate , pH. 5.2 was added, followed by 100μl of chilled ethanol and precipitated for 30min at -70 °C. The sample was spun at 10,000rpm for 10min at 4 °C. Supernatant was discarded and pellet was washed with ethanol, dried and dissolved in 7μl of sterile water.

b. Annealing of sequencing primer : 1μl of pUC sequencing or reverse sequencing primer was added to the template DNA. 2μl of 5x reaction buffer was added and incubated at 65 °C for 2min.

c. Labeling reaction : To the annealed template - primer. 1μl DTT (0.1M), 2μl labeling nucleotide mix, 5μCi of [α-^{35}S] or [α-^{32}P] dATP and 3.25 units of sequenase enzyme (version 2.0) were added. The sample was incubated for 2-5min at room temperature.

d. Chain termination : Four 1.5ml vials were labelled as G, A, T, C. 2μl of respective dideoxynucleotide mixture was taken in the four labelled tubes and warmed to 37 °C. 3.5μl of the template mixture was added to each of the tubes labelled as G, A, T, C and mixed and incubated at 37 °C for 5min. 4μl of formamide buffer was added to stop the reaction.

e. Casting and electrophoresis of sequencing gel : 6% denaturing polycacrylamide gel was cast as follows: 40g urea, 12ml of 40% polyacrylamide, 8ml of 10x TBE were mixed and volume was made to 80ml. The spacers used were 0.4mm thickness and the gel casting unit was 20cm x 60cm. The solution was degassed and 450μl of 10% freshly prepared ammonium persulphate and 60μl of TEMED (N,N,N'N'-tetramethyl ethylene diamine) were added and poured in the gel casting unit held by

tape and clamps. The comb was immediately inserted. After 30min, tapes were removed and gel was clamped to the electrophoresis unit which was maintained at 37 ^{0}C using circulation water bath. The samples were heated to 80 ^{0}C for 5min and were loaded and electrophoresed at 2000V till the bromophenol blue ran to one end of the gel. Samples were loaded for the second time and electrophoresis was continued till bromophenol blue touched the lower edge of the gel. Two loadings of the sample ensured more sequence reading. The gel was covered with saran wrap and dried on a vacuum gel dryer at 80 ^{0}C for 2hrs and exposed to X-ray film at -70 ^{0}C overnight.

Figure 21 shows the schematic representation of various steps involved in DDRT-PCR. The method consists of using sets of anchored and arbitrary primers to generate cDNA fragments by reverse transcription followed by polymerase chain reaction (RT-PCR). The cDNA fragments are resolved and compared in sequencing gels. The resulting cDNA patterns reflect differences in the mRNA composition. Differentially displayed cDNAs are isolated, cloned and sequenced. In this chapter, attempts were made to clone differential display cDNAs that are expressed by pathogen during process of infection in host plant.

Results

Identification of differentially expressed cDNAs

PCR based differential display technique made possible the amplification of the messenger RNAs which were present in very low levels and having four different sets of primers designed to capture total mRNA pool expressed at given time. Figure 22 reveals quality of total RNA isolated from pearl millet 7042-healthy, infected and resistant seedlings as well as fungal pathogen *S. graminicola* by presence of 28S and 18S bands prominently. These preparations were used for all the further analysis.
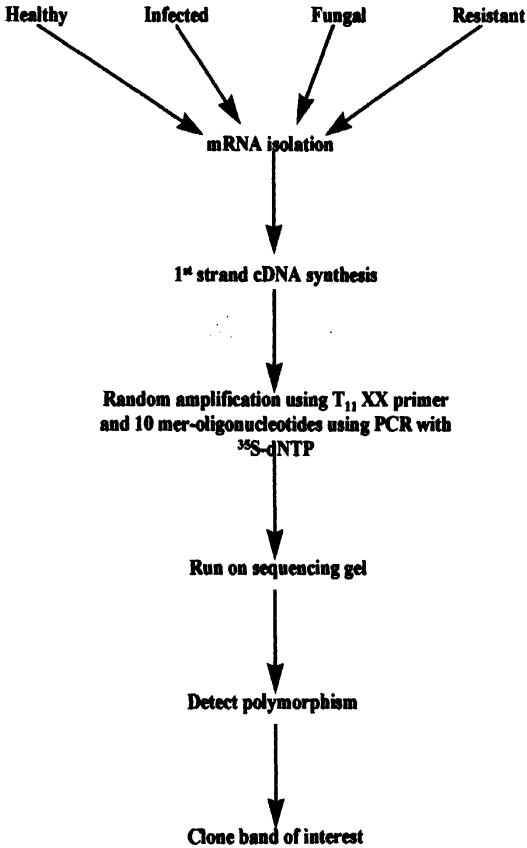
Fig. 21

Fig.21: Schematic diagram representing various steps in DDRT-PCR technique
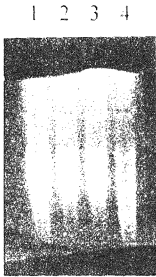
Fig.22
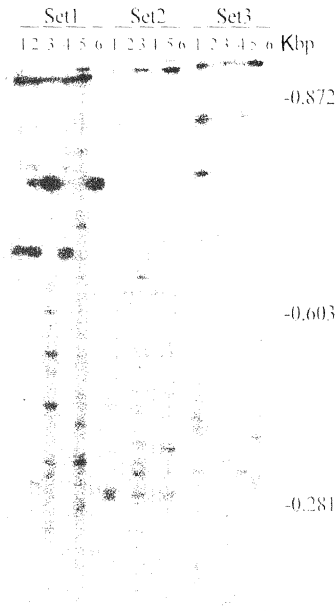
Fig.23

Fig.22 :RNA samples electrophoresed on formaldehyde agarose gel. Lane1 pearl millet healthy seedlings(7042S); lane2 pearl millet infected seedlings(7042S); lane3 pearl millet resistant seedlings (7042R) and lane4 *S.graminicola* (sporangia) RNA.

Fig.23 :A representative autoradiogram of differentially expressed PCR products with 3 different combinations of primers: Set1:T12MG with J18; Set2:T12MC with F3; and Set3:T12MT with F3. Each set of six lanes consists of 1-healthy; 2-infected; 3-infected control; 4-resistant; 5-resistant control and 6-fungal.
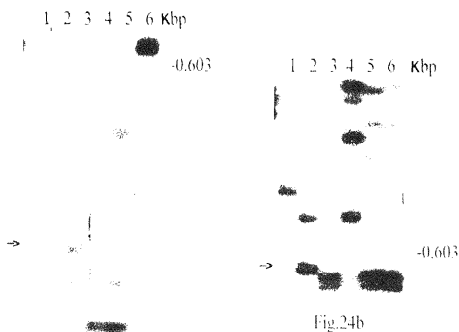
Fig.24a

Fig.24b
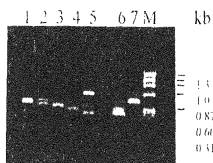
Fig.25

Fig.24a: Autoradiogram of differentially expressed PCR products with combination of T12MT and J18 primer set. Six lanes are of 1-healthy; 2-infected; 3-infected control; 4-resistant; 5-resistant control and 6-fungal. Note the specific amplifications of cDNA fragments in infected lane is indicated by arrows. Molecular marker is indicated on the right side of the margin.

Fig.24b: Autoradiogram of differentially expressed PCR products with combination of T12MC and F3 primer set. Six lanes consists of 1-healthy; 2-infected; 3-infected control; 4-resistant; 5-resistant control and 6-fungal. Note the specific amplifications of cDNA fragments in infected lanes is indicated by arrows. Molecular marker is indicated on the right side of the margin.

Fig.25 : A representative agarose gel showing PCR amplification of eluted bands from polyacrylamide gel. φx174 HaeIII digest molecular weight marker is indicated on right margin.

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To detect differences in mRNA populations of healthy, infected, resistant seedlings and fungal sporangia, first strand cDNAs were amplified by polymerase chain reaction with 60 Operon primers in combination with 4 sets of anchored primers. These PCR amplified products were size fractionated on polyacrylamide gels and were visualized by autoradiography.

Figure 23 is a representative autoradiogram of differentially expressed PCR products with three different combinations of anchored and Operon primers namely T12MG with J18, $T_{12}MC$ with E3 and $T_{12}MT$ with F3. As seen in this figure, apart from the common bands, there are several specific bands present either only in controls or in infected and resistant lanes. Three Operon primers E3, F3 and J18 have given consistent patterns in combination with three anchored primers and have also revealed the differences between four lanes of healthy, infected, resistant and fungal cDNAs (Fig.23). Bands that show up in 3rd (infected) and 5th (resistant) lanes were probably the result of PCR amplification from the contaminated genomic DNA in total RNA preparation and such bands were not considered. The PCR reactions in these lanes were without reverse transcriptase step, run along side for both infected and resistant samples.

Characterization of differentially expressed cDNAs

A total of 18 differentially displayed bands were selected from the autoradiogram in figure 23 for further characterization. Out of 18 bands, 12 were from infected-seedlings and 6 were from resistant-seedlings. All these bands were absent in healthy and sporangial lanes confirming their differentially expressed character. Two such differentially expressed bands from infected seedlings are shown clearly with an arrow in figures 24a and 24b. These bands were obtained with $T_{12}MT$ with J18 (Fig.24a) and $T_{12}MC$ with F3 (Fig.24b) primer combinations. All the differentially displayed bands, (total 18 in number) were eluted from the polyacrylamide gel and were further PCR amplified using same set of primer combination.

Fig.26a

Fig.26b

Fig.27a

Fig.27b
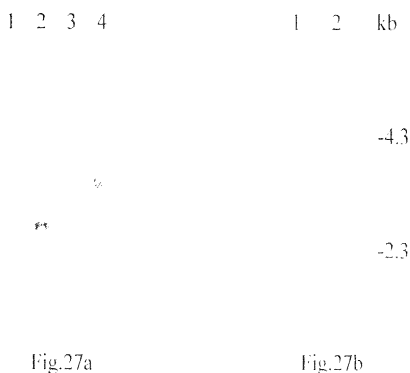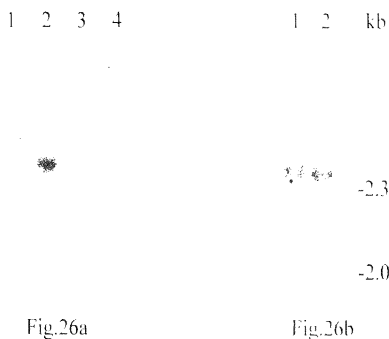
Fig.26a: Northern blot hybridization of clone *I*38 to total RNA of Pearl millet and fungus. Lane 1:healthy, lane 2:infected lane 3:resistant seedlings of pearl millet and lane 4:fungal sporangia of *S. graminicola*.

Fig.26b: Southern blot hybridization of clone *I*38 to genomic DNA of healthy pearl millet and of fungal sporangia digested with *BamHI* restriction enzyme. Lane1:healthy pearl millet seedlings(7042S); lane2:fungal sporangia and *λ. Hind*III digested molecular weight marker on right hand margin.

Fig.27a: Northern blot hybridization of clone *I*52 to total RNA of Pearl millet and fungus. Lane 1:healthy, lane 2:infected lane 3:resistant seedlings of pearl millet and lane 4:fungal sporangia of *S. graminicola*.

Fig.27b: Southern blot hybridization of *I*52 to genomic DNA of pearl millet seedlings and fungal sporangia digested with *BamHI* restriction enzyme. Lane1: healthy pearl millet seedlings (7042S); lane2:fungal sporangia and *λ. Hind*III digested molecular weight marker on right hand margin.

Figure 25 includes a representative agarose gel pattern of the amplified products of seven such bands. It is clear from this figure that PCR amplification of these eluted bands gives rise to 1 to 2 bands in the range of 300-600bp. All these bands were cloned using pMOS T-vector cloning kit (Amersham, UK) and were tested individually by hybridization on Northern blots containing RNAs from healthy, infected and resistant pearl millet seedlings and fungal sporangia. Clones which hybridized to all lanes on Northern blot were not processed further because they were not differentially expressed. Only two clones namely *I*52 and *I*38 showed differential hybridization on Northern and were obtained using the combination of Operon primer OPF3 & OPJ18 with $T_{12}MC$ and $T_{12}MT$ anchored primers, respectively.

Based on Northern data, these two clones can be considered as putative pathogen induced mRNAs from the infected host plant. To confirm the origin of these clones, they were hybridized with DNAs from the host plant as well as the infecting fungus. The clone *I*38 specifically hybridized to infected lane on Northern blot (Fig. 26a) and gave a single band with both fungal and plant DNA at the same molecular weight of around 2.3kb (Fig. 26b). The other clone *I*52 hybridized at higher molecular weight with fungal RNA and slightly at lower level with infected plant RNA (Fig.27a). Southern analysis (Fig.27b) showed two bands above 4kb size at same molecular weight with both fungal and plant DNA.

Discussion

Interactions between plant pathogens and their hosts involve a continual exchange of information between two organisms and provide an alternative system to study cell-cell communication, signal transduction and the regulation of gene expression in response to environmental stimuli. A proper understanding of the host-pathogen interaction would require elucidation of the bio-molecular pathways which are needed for either the pathogenesis or resistance.

In the present work an attempt has been made to use the technique of DDRT-PCR to identify cDNA clones which are induced in the host plant due to infection by pathogen. Ours is the first effort to carry out this type of work in an obligate system

where pathogen can survive only in the host-plant. The differential display has detected common bands between infected seedlings and fungal sporangia i.e. cDNAs from both fungus and plant. This is expected because in the host-pathogen interaction, genes from the host plant and pathogen are involved. The mRNAs expressed constitutively by house keeping genes from the fungus and the plant can be discriminated by comparison of the DDRT-PCR patterns of non infected plant (healthy) and fungus isolated *in vitro* (sporangia).

Presence of several specific bands in infected and resistant seedlings (Fig. 23) suggests the differential expression. In order to avoid selection of bands due to nonspecific amplification of traces of DNA in infected and resistant RNA samples, a control reaction committing the reverse transcriptase skip was run along with resistant and infected samples. Although PCR products could be observed at times in the control reactions, these products generally did not co-migrate with the bands observed in other lanes. The bands that showed up only in infected lanes or resistant lanes and absent in other lanes were selected to process further.

Southern hybridization of *I*52 at same molecular weight in lanes 1 & 2 (Fig. 27b) reveals that the gene is present in the fungus as well as in the host plant. In Northern, (Fig. 27a) the differences observed in hybridization patterns between infected and fungus were probably due to the result of size variation in transcript length which was thought to be very interesting. One hypothesis could be that the gene was expressed by the fungus and it has undergone post transcriptional modifications in infected host plant to carry out specific functions. The nucleotide sequence of clone *I*52 is given in figure 28 which did not show much significant homology to any known reported DNA sequence in data banks. Hence this may be a new stress related gene present in both fungus and plant. The clone *I*38 which was a differentially expressed transcript from infected plant also showed hybridization to both fungal and plant DNA (Fig. 26b). In Northern experiments, this clone hybridized specifically to infected plant RNA but not with fungal RNA (Fig. 26a). It appears that this transcript is probably expressed by the fungus in host plant to counteract host responses.

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TTTTTTTTTTT  TTGCGNATTC  ATTTCCCTTT  ATGNATCGCA
TCACCGCAAC   AGAAAAGCAA  CAACTACCAG  CTATTGCTTA
AGCTGTTAGA   ANANACGCGC  CTCCAACAGC  TTTGATTTTC
TTCTCAGCCT   CAGCTGANAA  GAACTTGCGC  TTAACAATCA
AAGGTACCTC   CGGCAGGCGA  CCCTTTCCAA  GCACCTTAAA
GTATCCAGCC   TTAGTTACGT  CAATAACAGG  GGCCTTGCCG
TCCTTCGATT   TTTCGGCCTG  CTCCTTGGTC  TTCTCAGACA
CTAGTGTCCA   TAAACGCTCC  ACATTTACCG  TANGGCAGTG
TTGGCGGTTT   CGCANCANAT  GGTAGTGTCC  ATACCAACTT
TACCAAAGTA   ACCCGGGTGG  AACTTGTGCA  TTAAAATACG
ATGGTGATGT   TGGCCACCAN  CGTTACCACG  ACCACCAGGG
TGCTTACGAT   GCTTGCCAAT  ACAACCGTGT  CCNGCACTCA
CATNGGCACG   TTTCTTGCGG  TTCTTGGAAA  AACNGGACGG
CATATTGACT   TNGTGATCAG  GA
```

Fig. 28. Nucleotide sequence of *I*52 clone

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Recently, Benito *et al.* (148) have used for the first time the DDRT technique to study plant-fungal pathogen interactions in tomato. They isolated cDNA fragments representing fungal genes whose expression was enhanced in plant and tomato genes in response to the infection caused by *B. cinerea*. Similarly Molina *et al.* (223) have identified two cDNAs encoding glycine rich proteins by DDRT approach whose mRNA levels increased with the infection of two fungal pathogens, *Erysiphe graminis* and *Rhyncosporium secalis*. Truesdell & Dickmann (147) have used differential display to isolate cDNAs related to plant defense – related proteins and tree pollen allergins from alfalfa on infection with the fungal pathogen, *Colletotrichum trifolii.*

In summary, DDRT-PCR offers an ideal approach for identification of differentially expressed transcripts during the process of infection. Identification and characterization of such differentially expressed genes will contribute to our understanding of the molecular basis of disease resistance in plants. Finally engineering important crop plants with these genes will increase their resistance to phytopathogenic organisms.

CHAPTER-VI

OVERVIEW AND FUTURE PERSPECTIVES

Downy mildew disease of pearl millet is highly devastating because it causes damage to the panicle, an important plant part and due to the survival of oospores for a long time in soil,. Although this pearl millet disease is economically important, very little attempt has been made to study the pathogen using molecular approaches and its host specificity. Such data will form a strong base to plan further strategies to breed pearl millet with more durable downy mildew resistance. In my thesis work, I have focused the attention on the following aspects of the pathogen:

- To carry out basic genome analysis with special emphasis to repeat elements, retrotransposable elements and ploidy levels.
- To study genetic variability using different DNA markers such as minisatellites and microsatellites.
- To gain insight into host specificity and adaptability, using cross inoculation experiment.
- To identify pathogen-induced expression in host plant using DDRT-PCR approach.

Genome analysis

The basic genome analysis of the fungus has given information about the content of repetitive DNA, presence of retrotransposable elements and ploidy levels. Repetitive elements are responsible for generation of variability as they are believed to be the hot spots for genetic recombination (224). For example, Hammer *et al.* (65) have reported the host species - specific conservation of a family of repetitive sequences in the genome of *Magnaporthe grisea* pathogen. Identification of retrotransposon like elements in the genome of *S. graminicola* is another important observation supporting ample genetic diversity. There is a direct evidence of retrotransposon sequence rearrangement in the genome of *Fusarium oxysporum* due to stress conditions (225). The ploidy differences in *S. graminicola* represent another important observation which is an advanced character observed in this class of Oomycete fungi.

Population dynamics of *S. graminicola*

The population studies using DNA fingerprinting have detected considerable variation in *S. graminicola*. Eventhough host specific isolates are from the same area e.g. Patancheru, they show lot of variation among them which is probably due to the kind of cultivars grown in particular areas. This is also revealed by the DNA fingerprinting pattern in zoospore and oospore isolates. The studies of population dynamics are centred around the potential variation present in the populations and the mechanisms by which changes in the virulence occur. In asexual populations, observation of such large variation is very interesting. Generation of such variation either in asexual or sexual reproduction needs to be further studied. Genetic markers will be of great help to determine the mechanisms by which polymorphisms are generated.

Converting hybridization based markers to PCR based markers for rapid and easy monitoring of the ever changing, dynamic pathogen, would serve as a simple and efficient diagnostic tool. Development of PCR based markers for two mating types PT2 and PT3 in order to generate molecular genetic map for downy mildew pathogen will be another development in this area.

Fingerprints generated by PCR will be more advantageous than DNA fingerprints based on Southern hybridization, which involves difficulties such as slow experimental process, use of radioactivity in Southern hybridization, limitation in number of samples that can be analysed at a time and large quantities of DNA for repeated analysis.

Host adaptation and specificity

Fungal plant pathogens grow preferentially or exclusively on a limited number of hosts. Therefore, it can be suggested that some factors may exist which restrict the host range of pathogen. Relatively little is known about the interactions that occur between pathogenic fungi and their host plants and about the changes that occur in fungal pathogen populations over a certain time span. Our cross inoculation experiments show the slow and steady adaptability of downy mildew pathogen to new pearl millet cultivars in course of time. Our experiments have revealed that it

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took 10 generations (roughly equal to 3 years or 6 seasons) to adapt to new cultivar. Repeated cultivation of the same cultivar for few seasons in a particular area eventually builds up or selectively enriches the particular virulent race. Change in cultivar, therefore, may show resistance initially but becomes susceptible after a certain time span. The selection pressure imposed on the pathogen may result in significant changes in avirulence and virulence genes thereby breaking the resistance of changed cultivar. As our knowledge of both host and pathogen improves, it will be exciting to learn how pathogens evolve strategies for colonising on specific hosts. Controlled crossing in a laboratory is a prerequisite for dissecting the genetic basis of host species and cultivar specificity. In *S. graminicola* such methods need to be standardised to study genetics of host specificity.

Further studies need to be carried out to understand the genetic basis of host specificity with new set of genotypes and homogeneous single oospore and zoospore derived pathotypes.

Defence responses from host plants

Subtraction techniques such as DDRT-PCR can help in identifying the molecular basis of disease. The complex communication involved between virulent pathogen and recognition of susceptible host and avirulent pathogen and resistant host can be studied using this method by sampling at different time points. It provides an alternative strategy for identification of resistance genes apart from laborious methods such as map based cloning and chromosome walking. Identification of resistance genes for downy mildew has started unravelling such complex mechanisms in *Arabidopsis* (158). These genes can be used as probes to screen genomic and cDNA libraries to isolate related gene families in heterologus systems or can be amplified in PCR using degenerate oligonucleotide primers. These resistance genes will have immediate utility in plant genetics and selection and will represent the true molecular markers for disease resistance. These will be useful for germplasm analysis and indirect selection of resistance in the absence of pathogen. Isolation of resistance gene families is a major step towards transgenic strategies for disease control. Resistance gene markers can be used to screen for novel R genes in

germplasm. It will be possible to generate cassettes of transgenes that can be manipulated as a single Mendelian unit in classical breeding programs (226).

DDRT-PCR products isolated from infected seedlings can be further characterised to determine their role in the infection process, which will open the avenue of genetic engineering to develop resistant cultivars.

Downy mildew disease will continue to be a threat to pearl millet. Introduction of resistant cultivars is the most successful and economical way to overcome downy mildew in India and also in Africa. Continuous monitoring of the pathogen population using modern molecular tools is needed to detect the development of new virulent races. This complements the efforts to develop new resistant cultivars with different genetic base to face threats of shifts in pathogen population. Strategies for efficient utilisation of resistance genes that include pyramiding several genes and deploying different genes over a period of time will have to be developed. These strategies will restrict the development of virulent populations for a longer time and provide durable control of the disease in the pearl millet growing areas. Knowledge generated by understanding pathogen population and resistant cultivar development should be complemented with new cultural practices.

THESIS ABSTRACT

An introduction to downy mildew host-pathogen relationship and its molecular characterization

Pearl millet (*Pennisetum glaucum* (L) R. Br.), the most drought tolerant of all domesticated cereals, can grow on the poorest soils under harsh climatic conditions and still yields reasonably well where most other crops fail. It is widely distributed across the semi arid tropics of Africa and Asia and is the principal food crop across sub-Saharan Africa and North western India. It is planted on some 15 million ha in Africa and 10 million in Asia yielding approximately 10 million tons of grain.

One of the major yield reducing biotic factors in pearl millet, is the disease downy mildew caused by *S. graminicola* (Sacc.) Shroet, an obligate, oosporic biotroph. Severely infected plants remain stunted, and green ear symptoms appear on panicles due to transformation of floral parts into leafy structures.

Until recently, the downy mildew pathogen has received very little attention due to difficulties of maintaining the isolates on the living host plants. Although the plant breeders have identified many genes conferring resistance to downy mildew pathogen, these genes have been rendered ineffective due to variability in the pathogen population. An in-depth knowledge of pathogen as well as host-pathogen interaction is, therefore, essential to understand the mechanism of variability in the pathogen population. Such a study will possibly enable the breeders to devise strategies to control the disease.

Thesis Project.

In 1992-93, a collaborative research effort involving National Chemical Laboratory, Pune and International Crops Research Institute for Semi Arid Tropics, Hyderabad was initiated to carry out studies on the downy mildew pathogen with following objectives.

- To get basic knowledge of the pathogen DNA.
- To assess the genetic variability of the pathogen
- To gain insight into some aspects of host-pathogen interaction.

The important research findings under each objective are summarized in the following paragraphs

Characterization of the genome of *S. graminicola*, the causal fungus of downy mildew of pearl millet.

1. When DNA from *S. graminicola* was digested with hexa-cutter and tetra-cutter restriction enzymes a large number of bands appeared on ethidium bromide stained agarose gels indicating the presence of repeat elements.

2. A genomic library of the most virulent pathotype 7042S of *S. graminicola* was constructed in lambda gt11. Repetitive DNA content in *S. graminicola* was estimated based on hybridization of genomic DNA library clones. The results indicated that the total repetitive DNA content was about 8% of which 2% was highly repetitive and 6% moderately repetitive. Repeat elements on Southern analysis revealed that they were dispersed in the genome of the fungus.

3. Digestion of DNA with isoschizomeric methylation-sensitive enzymes, *Dpn*I and *Mbo*I showed that adenine nucleotide in GATC sequence of the genome was partially methylated, and that there was no cytosine methylation. The specific variation in electrophoretic pattern of repeat elements in the genome digested with methylation sensitive enzymes indicated predominance of A methylation, and CpG, CpC methylation in GATC and CCGG containing sequences, respectively.

4. The presence of retrotransposable elements was tested using a set of primers based on conserved sequences of reverse transcriptase. The P5 probe from the conserved region of the reverse transcriptase of *Fulvia fulva* showed hybridization with PCR amplified products from *S. graminicola* suggesting presence of retrotransposon-like elements in the fungal genome.

5. Cytofluorometric analysis of sporangia revealed differences in ploidy level in sporangial population of haploids, diploids and triploids where haploids and triploids were predominant over diploids. These ploidy differences could be important in establishing population genetic diversity.

Genetic variability analysis in *S. graminicola* using DNA markers

1. The potential of several approaches including a reliable and versatile technique of DNA fingerprinting and other repeat DNA sequences was examined to study the extent of genetic variation in six host specific pathotypes of pearl millet downy mildew pathogen. Simple repetitive DNA sequences such as $(GATA)_4$, $(GACA)_4$, $(GAA)_6$, $(CAC)_5$, $(GGAT)_4$, and $(TG)_{10}$ were tested for their ability to detect polymorphism in *S. graminicola* pathotypes using several restriction enzymes. Detection of a high level of polymorphism among the six host-specific pathotypes using $(GATA)_4$ was unique and the other repeat probes did not bring about this level of polymorphism with any of the restriction enzymes used in the study. $(GATA)_4$ could generate pathotype specific fingerprint pattern for six host-specific pathotypes. Microsatellite $(GATA)_4$ - derived DNA fingerprints were stable even after ten asexual generations, indicating the utility of this probe in DNA fingerprinting.

2. Dendrogram based on DNA fingerprints obtained with all three probes $(GATA)_4$, $(GACA)_4$ and $(GAA)_6$ clustered the six pathotypes into five groups.Path-5, Path-3 and Path-4 formed distinct groups, whereas Path-1, Path-2 and Path-6 together formed a separate group in which Path-1 and Path-2 clustered as a subgroup and Path-6 another.

3. Since $(GATA)_4$ probe could decipher DNA fingerprints with host-specific pathotypes, this probe was further used to analyze the oospore and zoospore derived isolates collected from different infected fields in India. The isolates showed diverse DNA fingerprint patterns which were unique compared to the six host-specific pathotypes. Further, the variation/similarity between individual isolates and different zoospore isolates collected from the same location was also studied. All the three zoospore isolates obtained from Coimbatore showed considerable variation. On the other hand, the oospore isolate CBOS1 and zoospore isolate CBOS1Z-1 showed almost similar patterns. Interestingly, PT3 isolate showed almost 100% similarity with Mysore oospore isolate MYS OS1.

Genetic basis of adaptability of *S. graminicola* to individual host genotypes of pearl millet

Host adaptability between cultivar-pathotype combinations was examined using host-pathogen swap experiments. Two pearl millet downy mildew pathotypes specific to different pearl millet cultivars were cross inoculated and maintained for 10 asexual generations to test their adaptability. The virulence and latent period of infection were measured at alternate generations on their respective hosts. The pathogen populations from the infected seedlings were screened with RAPD markers and SSRs to detect changes at the genomic level of the pathogen. DNA fingerprinting profile obtained after 10 generations showed differences with the microsatellite probe $(GATA)_4$. Pathological studies also revealed increase in virulence on the new cultivar and a relative decrease in virulence pattern on the earlier host-cultivar. These results support the idea of genetic adaptation of the fungus to the individual host species.

Assessment of pathogen induced host plant response using DDRT-PCR approach

In order to isolate genes that are induced and expressed during the process of infection, seedlings of pearl millet were inoculated with fungal spores at first leaf stage and the tissue was harvested 6 days later when symptoms of infection could be observed. Total RNA was isolated from the healthy, infected, and resistant seedlings and fungal sporangia and was analyzed by the RT-PCR method. Differentially expressed cDNA bands present only in infected and resistant seedlings but absent in healthy and fungal sporangia were isolated. These bands were reamplified, cloned and hybridized to Northern blots for confirmation. The fragments that showed positive results were sequenced and further characterized.

Publications

1. DNA fingerprinting detects genetic variability in pearl millet downy mildew pathogen *Sclerospora graminicola* (1995)
   JG Sastry, W. Ramakrishna, S.Sivaramakrishnan,R.P Thakur, Vidya S. Gupta, P.K Ranjekar Theor. Appl. Genet. 91:856-861.

2. Genome characterization of *Sclerospora graminicola* the causal fungus of downy mildew of pearl millet (1997)
   JG Sastry, S.Sivaramakrishnan, R.P Thakur, Vidya S. Gupta, P.K Ranjekar. World Journal of Microbiology and Biotechnology 13:693-697.

3. Genetic basis of host-specificity in pearl millet downy mildew pathogen (*Sclerospora graminicola*)
   JG Sastry, S.Sivaramakrishnan, R.P Thakur, Vidya S. Gupta, P.K Ranjekar. (Communicated to Journal of Phytopathology)

4. Identification of differentially expressed mRNAs in pearl millet-downy mildew pathogen (*Sclerospora graminicola)* system using DDRT-PCR .
   JG Sastry, S.Sivaramakrishnan, R.P Thakur, Vidya S. Gupta, P.K Ranjekar (Under preparation)

Workshops and conference presentations

1. DNA fingerprint detects genetic variability in pearl millet downy mildew pathogen *(S. graminicola)*
   JG Sastry, W. Ramakrishna, S.Sivaramakrishnan,RP Thakur, VS. Gupta, PK Ranjekar. (poster presented during 16th International union of Biochemistry and Molecular Biology Conference at New Delhi, India 19th-22nd September 1994).

2. Biotechnological applications in pearl millet downy mildew research
   N. Seetharama, JG. Sastry and HS. Shetty (brochure presented during workshop on Pearl Millet Downy Mildew, 18-22 March 1996 at IAC, Patancheru, AP. 502 324)

3. Molecular studies on genetic variability and plant-pathogen interaction studies in pearl millet downy mildew fungus (*(S. graminicola)*.
   Renuka Singru, JG Sastry, S.Sivaramakrishnan, RP Thakur, VS. Gupta, PK Ranjekar.(A Poster presented during the international conference on integrated plant disease management for sustainable agriculture 10-15 Nov 1997, India)

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