“COMPARATIVE TRANSCRIPTOME ANALYSIS FROM RESISTANT AND SUSCEPTIBLE PEARL MILLET (*Pennisetum glaucum* L.) GENOTYPES IN RESPONSE TO DOWNY MILDEW (*Sclerospora graminicola* Sacc.) INFECTION”

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ABSTRACT

Pearl millet (*Pennisetum glauccum* (L.) R. Br.) is an annual, cross pollinating, monocot crop belonging to the family Poaceae and sub family Penicedae. It is the sixth most important cereal of the world and has critical value as food security in some of the world’s hottest and driest cultivated areas of Africa and Asia. However, the crop faces constraints to the productivity which includes the prevalence of different biotic and abiotic stresses. Among them, the major biotic constraint in pearl millet production is downy mildew disease caused by the pathogen *Sclerospora graminicola* (Sacc.) which have a great negative impact on the Indian economy with an estimated yield loss of up to 40–60%. The present work was carried out with an aim to find out the candidate genes responsible for resistance in pearl millet against downy mildew and comparative transcriptome analysis from resistant and susceptible pearl millet (*Pennisetum glaucum* L.) genotypes in response to downy mildew (*Sclerospora graminicola* Sacc.) infection. The current study helped

to catalyze the novel purpose of transcriptomic data of pearl millet against downy mildew for the first time and could provide impetus for furtherance of research in the pearl millet-downy mildew infection.

Two days old grown seedlings of downy mildew resistant (J2290) and susceptible (7042S) genotypes were inoculated with *S. graminicola* inoculum (inoculated) and water (control). The seedlings were observed for hypersensitive response which was rapid in resistant inoculated seedlings with necrosis appearance as early as six hours post inoculation (hpi) followed by susceptible inoculated seedlings which showed necrosis after 18 hpi. Peroxidase activity was measured and resistant inoculated showed increased peroxidase activity than resistant control, susceptible inoculated and susceptible control seedlings. A correlation between disease reaction in form of HR and peroxidase activity was observed and HR served as a morphological marker of resistance while peroxidase served as biochemical marker of resistance in pearl millet against downy mildew infection.

For transcriptome analysis, total RNA was isolated from all the four different treated seedlings and evaluated for quality and quantity. This was followed by mRNA isolation and its fragmentation which was quality checked on Bioanalyzer. Double stranded cDNA was synthesised and Multiplex Identifier adapters were ligated, thus generating four libraries. The cDNA libraries were quality, quantity checked on Bioanalyzer and Fluorometer. The cDNA libraries were clonally amplified through emulsion PCR and amplified fragments were sequenced on the 454 GS FLX Pyrosequencer.

The 454 pyrosequencing sequencing generated 34 Mb data for all the four multiplexed samples with a total of 134788 raw reads (34890767 bases). The maximum read length was 604 bases with an average read length of 258 bases. The reads utilized for generating unigenes for all the four libraries were 32596, 46272,

21606, 23135 and the number of unigenes generated were 2139, 2046, 1425 and 694 respectively.

The analysis of sequencing reads were bifurcated to three tiers which included candidate genes involved in resistance in pearl millet against downy mildew infection, transcripts specifically and differentially expressed in resistant and susceptible inoculated and control; within the resistant and susceptible inoculated and control genotypes. Specific and differential expression of genes in response to downy mildew infection was observed in the genotypes. Overall, it could be concluded that the resistant and susceptible genotypes responded to downy mildew infection by activating resistance responses. The plants tried first to wedge the progression of the pathogen by increasing the expression of hydrolases and cell death, culminating in the form of hypersensitive response. The infection was also sensed by various kinases like MAPK, serine/threonine kinases, shaggy kinases mediated through various secondary messengers like Ca^{2+} which activated other transcriptions factors such as Myb, F-box, DEAD box, heat shock transcription factors, DREB, creb and WRKY, which in turn induced the expression of defense related enzymes from the phenylpropanoid, shikimate pathway and other pathways that are involved in resistance to pathogens. The other category of genes that seemed to be involved in plant resistance to the pathogen encoded proteins involved in lignin biosynthesis such as Caffeoyl-CoA O-methyltransferases, Cytochrome P450, lipases, isochorismate synthase, chalcone synthase and S-adenosylmethionine synthase. The genes involved in resistance pathogenesis related proteins and certain specific genes like *mlo*, *edr*, stress enhanced proteins, *Avr9 cf* proteins were bestowed in providing resistance to downy mildew infection in pearl millet.

The sequences generated from downy mildew resistant and susceptible genotypes covered various biological processes and molecular functions indicating that 454 sequencing constituted a powerful tool for sequencing the transcriptome. The dynamics of

genes involved in resistance were revealed in pearl millet against downy mildew infection through transcriptome analysis of pearl millet. Furtherance of the present work could be envisaged by carrying annotation of unknown reads from the transcriptomic data and its functional validation at proteomics level.

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CERTIFICATE

This is to certify that the thesis entitled **"COMPARATIVE TRANSCRIPTOME ANALYSIS FROM RESISTANT AND SUSCEPTIBLE PEARL MILLET (*Pennisetum glaucum* L.) GENOTYPES IN RESPONSE TO DOWNY MILDEW (*Sclerospora graminicola* Sacc.) INFECTION"** submitted by **KALYANI S. KULKARNI** in partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** in **AGRICULTURAL BIOTECHNOLOGY** of the Anand Agricultural University is a record of bonafide research work carried out by her under my personal guidance and supervision. The thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Place: Anand **(R. Bhatnagar)**
Date: 14/07/2011
Major Advisor

DECLARATION

This is to declare that the whole of the research work reported here in the thesis for the partial fulfillment of the requirements for the degree of **Master of Science** in **Agricultural Biotechnology**, by the undersigned is the results of investigation done by her under the direct guidance and supervision of **Dr. R. Bhatnagar,** Research Scientist and Head, Department of Biochemistry, B. A. College of Agriculture, Anand Agricultural University, Anand and no part of the work has been submitted for any other degree so far.

Place: Anand
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HUMAN HEART IS HAVING ITS OWN REASONS THAT REASON KNOWS NOTHING

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ABBREVIATIONS

%	Percentage
µl	micro litre
^{0}C	Degree Celsius
AFLP	Amplified fragment length polymorphism
ATP	Adenosine trinucleotide phosphate
avr	avirulence
B2G	Blast2GO
BDD	Bead Deposition Device
bp	Base pair
C.D.	Critical difference
C.V.	Coefficient of variation
CAT	Catalase
CBP	Calcium-binding protein
CCaMK	Calcium and calmodulin dependent protein kinase
cDNA	Complementary deoxyribose nucleic acid
COS	Chitooligosaccharide
CRG	Chitin responsive gene
CWF	Composite wells format
DAG	Days after germination
DM	Downy mildew
DNA	Deoxyribose nucleic acid
DTT	Dithiothreitol
emPCR	emulsion PCR
EREBP	Ethylene responsive element binding protein
ET	Ethylene
FAO	Food and Agricultural Organization of United Nation
FLcDNA	Full length cDNA
GO	Gene Ontology

GS FLX	Genome Sequencer FLX System
h	hours
ha	Hectare
hpi	Hours post inoculation
HR	Hypersensitive Response
HRGP	Hydroxyproline rich glycoproteins
HSP	Heat shock protein
IAAO	IAA oxidase
IDA	Inferred from direct assay
IEA	Inferred from electronic annotation
ISSR	Interspersed simple sequence repeat
JA	Jasmonic Acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
Kg	Kilo gram
LCM	Laser capture microdissection
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LVE	Large volume emulsion
MAMP	Microbial-associated molecular pattern
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MID	Multiplex Identifier
ml	mili litre
Mm	Mili meter
MPSS	Massively Parallel Sequencing by Synthesis
mRNA	messenger RNA
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide Hydrogenase
NBS-LRR	Nucleotide Binding Signal- Leucine Rich Repeat

ng/l	nanogram per litre
nm	nano meter
PAL	Phenylalanine ammonia lyase
PAMP	Pathogen-associated molecular pattern
Pg	Picogram
PMPF	Pre-mRNA processing factor
POX	Peroxidase
PPO	Polyphenol oxidase
PR	Pathogenesis related proteins
PRR	Pattern recognized receptor
PTP	Pico Titer Plate
PVP	Polyvinylpyrolidone
qPCR	Qualitative Polymerase Chain Reaction
R	Resistance
RAPD	Randomly amplified polymorphic DNA
RCA	Reviewed computational analysis
RH	Relative humidity
RLK	Receptor-like kinase
RNA	Ribonucleic acid
RNA	Ribose nucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT	Real Time
RUBISCO	Ribulose bisphosphate carboxylase
SA	Salicylic acid
SAGE	Serial analysis of Gene Expression
SAM	S-Adenosyl-L-methionine
SAR	Systemic Acquired Resistance
SBS	Sequencing-by-synthesis

SCAR	Sequence cleaved amplified region
SEm	Standard error of mean
SFF	Standard Flowgram Format
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SOLiD	Sequencing by Oligonucleotide Ligation and Detection
SSR	Simple sequence repeat
SVE	Small volume emulsion
T	Temperature
t	Ton
TE	Tris EDTA
TF	Transcription factor
TIGR	The Institute of Genomic Research

I. INTRODUCTION

Pearl millet (*Pennisetum glauccum* (L.) R. Br.) is an annual, cross pollinating, monocot crop belonging to the family Poaceae and sub family Penicedae. It is a high-yielding, diploid (2n=2x=14) C4 summer grass with a haploid genome size of 2350 Mbp and DNA content of 1C = 2.36 pg (Budak *et al*., 2003). It is indigenous to the areas of North Africa and is primarily grown as a rainfed crop in the low rainfall zones of Sub-Saharan Africa and the Indian subcontinent. It is known by various names in different languages: pearl, bulrush, cattail, or spiked millet in English; *bajra* in Hindi; *dukhn* in Arabic; and *mil chandelles* in French and is cultivated as a cereal or forage. Pearl millet is the sixth most important cereal in the world, including pseudocereals. It is considered as a poor man's crop, as it is grown in low-input, rain-fed agricultural systems where farmers adopt only seed treatment practices for managing pests and diseases (Nithya *et al*., 2007).

Globally, pearl millet is cultivated in an area of 35.15 m ha with an annual production of 28.52 Mt and an average yield of 811 kg/ha. India is the largest producer of pearl millet, both in terms of area (8.74 m ha) and production (8.83 Mt), its yield being 1011 kg/ha. In India, pearl millet ranks third in terms of area, fourth in terms of production and sixth in terms of yield among the cereals. Principle states in India, under pearl millet cultivation are Rajasthan, Maharashtra, Gujarat and Punjab. It is sown as a winter crop in the eastern side of the Western Ghats and in Tamilnadu (George *et al.,* 2005). In Gujarat, it is cultivated over an area of 0.92 m ha, with the production of 1.31 M t and yield of 1419 kg/ha.

Pearl millet is of critical value for food security in some of the world's hottest and driest cultivated areas of Africa and Asia (Singh *et al*., 1993). Nutritionally superior to rice and wheat, pearl millet is commonly baked as unleavened bread, or cooked as thin or thick

porridge (Sawaya *et al*., 1984). Millets typically contain higher quantities of essential amino acids methionine and cysteine and are higher in fat content than maize, rice, wheat, and sorghum (Obilana and Manyasa, 2002).

The major biotic constraint in pearl millet production is downy mildew (DM) disease caused by the pathogen *Sclerospora graminicola* (Sacc.) which have a great negative impact on the Indian economy (Singh, 1995). Estimated yield loss of up to 40–60% has been recorded (Hash *et al*., 2003). The fungal pathogen, *Sclerospora graminicola* causing downy mildew disease of pearl millet belongs to the family Peronoporaceae in the class Oomycetes. It is an important pathogen on pearl millet, prevalent wherever the crop is cultivated in the semi-arid tropics. Two types of symptoms namely downy mildew and green ear are produced with various types of proliferations and malformation of the panicle. The disease incidence positively correlates with relative humidity and maximum temperature.

Plant defense mechanisms operate through the activation of multiple defense proteins. In fungal plant pathogenesis, enzymes play a crucial role through external and internal interactions to resist the development of fungal pathogens. Enhanced biosynthesis and activity of some enzymes are the most important processes in plant defense in specific plant-fungal pathogen interactions (Thukral, 1986). The synthesis of new proteins that have direct or indirect action on the course of pathogenesis have the major effect on plant resistance to a given pathogen (Manjunatha *et al*., 2008). These proteins include a heterogeneous group of proteins collectively defined as the Pathogenesis-related (PR) proteins (Van Loon, 1985). Among these PR proteins, hydrolases such as β-1, 3-glucanases and chitinases, chitosanases and peroxidases have been implicated in the resistance against fungal pathogens of plants against potential pathogens (Kini *et al.,* 2000).

The host-pathogen interaction in the pearl millet-DM system is expected to follow the general gene-for-gene concept (Flor, 1971). The gene-for-gene relationship envisions that dominant resistance genes interact with fungal avirulence genes, which usually results in a hypersensitive response (HR). HR is one of the most frequently occurring defense responses in crop plants against pathogens, such as viruses, bacteria, fungi and nematodes. Resistance conferred by a single dominant allele is expressed in seedlings as well as in adult plants and is characterized by a hypersensitive reaction type due to accumulation of some defense compounds (Thakur and Mathur, 2002).

Many genes contribute to downy mildew resistance, these genes are scattered throughout the host genome, pathogen-strain specificity is the rule for each of these genes and a large portion of resistance to a given pathogen population can be accounted for by relatively few genes (Anon, 1996). The study of resistance is complicated since both the host (Brunken, 1977) and pathogen (Idris and Ball, 1984) are out-breeding, and are highly variable exhibiting non-Mendelian segregation ratios for host plant resistance. Studies on downy mildew are complicated by the fact that it cannot be grown as an axenic culture in laboratory (Singru, 2003). Details of breeding for disease resistance in pearl millet have suggested the need of knowledge of genetic structure of the host and pathogen populations for durable resistance against downy mildew (Andrews *et al*., 1985; Hash *et al*., 1997; Jeger *et al*., 1998).

Transcriptomics is the quantification of the transcriptome, the complete set of transcripts in a cell and their abundance, for a specific developmental stage or physiological condition (Wang *et al*., 2009). The field of transcriptomics focuses on such studies, where experiments are designed to monitor and manipulate the dynamics of gene expression events that occur during both resistance and susceptibility to particular stresses (Coram *et al*., 2008). Transcriptome sequencing is a convenient way to rapidly obtain

information on the expressed fraction of the genome providing an unbiased representation of all regions of a transcript, independent of length or expression level. The Genome Sequencer FLX System (GS FLX), powered by 454 Sequencing, is a next-generation DNA sequencing technology, featuring a unique mix of long reads, exceptional accuracy and ultra-high throughput. It is an emerging platform for *de novo* sequencing of transcriptomes or genomes in order to easily analyze and annotate the uncharacterized genomes (Cheung *et al*., 2008). The 454 pyrosequencing promises a ~100-fold increase in throughput over Sanger technology which is advancement to make large and complex genomes more amenable to full genome sequencing at affordable costs.

Studies of changes in the host transcriptome (Wise *et al*., 2007) or proteome (Xing *et al*., 2001; Mehta *et al*., 2008) during infection by a range of pathogens suggests that hundreds of plant genes are up or down regulated during the response. These include genes involved in pathogen recognition (Takken *et al*., 2006), signal transduction (Beckers and Speol, 2006), defense response as well as genes involved in the redirection and recruitment of energy (Fritig *et al*., 1998).

Transcriptomics has had a significant role in improving the understanding of fungal plant diseases (Tan *et al*., 2009). Transcriptome analysis can throw insights into the underlying mechanism of resistance in pearl millet in response to downy mildew infection. The transcriptome of pearl millet is not yet sequenced and sequencing can reveal and unravel many facets of expression which can be further extended to the molecular and breeding levels. Hence, the above study was undertaken keeping in view the following objectives:

1. To find out the candidate genes responsible for resistance in pearl millet against downy mildew.

2. Transcriptome comparison between infected and control tissues within susceptible and resistant genotypes.

3. Transcriptome comparison between infected and control tissues of susceptible and resistant pearl millet genotypes.

II. REVIEW OF LITERATURE

2.1 Introduction

2.1.1 Origin of Pearl millet

Pearl millet (*Pennisetum glaucum* L.) is the most widely grown type of millet. Pearl millet was originated in Africa and subsequently introduced in India.

2.1.2 Importance of Pearl millet

The crop is primarily cross pollinated, and following pollination, it takes a flower about 30 more days to develop into a mature seed. Pearl millet appears to have relatively fast root development, sending extensive roots both laterally and downward into the soil profile to take advantage of available moisture and nutrients. It also tolerates low soil pH better than other crops. The contribution of pearl millet in terms productivity to the food basket is comparatively high. They are grown with limited water resources and usually without application of any fertilizers or other inputs by a multitude of small-holder farmers in many countries. Therefore, because they are mostly consumed by disadvantaged groups, they are often referred to as "coarse grain" or "poor people's crops". The ovoid grains are about 3 to 4 mm long, much larger than those of other millets, and the 1000-seed weight ranges from 2.5 to 14 g with a mean of 8 g. The size of the pearl millet kernel is about one-third that of sorghum. The relative proportion of germ to endosperm is higher than in sorghum (FAO, 2004).

2.2 Nutritional importance

Pearl millet has nutritionally rich carbohydrates (72%), protein (19%) and fat (4.6%). Pearl millet contained 88–91% dry matter, 1.6–

2.4% ash, 2.6–4.0% crude fiber, 2.7–7.1% oil, 8.5–15.1% crude protein, 58–70% starch and 354–796 mgg^{-1} phytic acid. Mineral contents were 10–80, 180–270 and 450–990 mgg^{-1} Ca, Mg and P, respectively, and 70–110, 4–13, 53–70, 18–23, 10–18 and 70–180 μgg^{-1} K, Na, Zn, Mn, Cu and Fe respectively (Abdalla *et al*, 1998). Pearl millet contains higher quantities of essential amino acids like lysine, methionine and cysteine and is comparatively high in protein with a good amino acid balance (Obilana and Manyasa, 2002). Pearl millet contains 27-32% more protein than maize, and also higher protein and fat than wheat and rice. The grain is also comparatively high in fat, and linolenic acid comprises 4% of the total fatty acids. Pearl millet oil contains more palmitic, stearic, and linolenic and less oleic and linoleic fatty acids than corn oil. The energy density of pearl millet is relatively higher arising from its high oil content, relative to maize, wheat or sorghum. It is also digested slowly and so staves off hunger for longer period (Gill and Turner, 2001). It contains similar amounts of Ca and P (Burton *et al*., 1972) and particularly rich in zinc and iron relative to these cereals (Khairwal *et al*., 1997). Trace mineral contents of pearl millet are two to ten times higher than that of rice (Agte *et al*., 2005). Protein storage fraction consists of the alcohol soluble prolamins. These prolamins called pennisetins comprise 33.1-49.5% of the total protein fraction (Okoh *et al*. 1985). The anti-nutritional factors present in pearl millet are tannins and phenols (Nithya *et al*., 2007). Even when grown in highly stressed conditions, the grain is essentially free of aflatoxins and fumonisins.

2.3 Cultivation practices

Pearl millet is the most widely cultivated and investigated of all millets (Babatunde and Manyasa, 2002). It is a short-day plant, adapted to warm, semi-arid, desert climates. Pearl millet is grown

where no other cereal can yield grain, in regions with 200-800 mm of annual rainfall. Pearl millet cultivation is dispersed mainly during Kharif (rainy) season across the country. It is also grown to a lesser extent during Rabi (post-rainy) season in Andhra Pradesh, Karnataka, Tamil Nadu and Pondicherry. It is also grown during summer season in Punjab and Rajasthan; the yield of pearl millet varies from state to state with varying rainfall and soil type, and also between seasons. Only about 8% of pearl millet area is irrigated. Summer pearl millet is popular in Gujarat state with very high yield exceeding 2.03 t/ha with excellent grain quality (Anon, 2009). The height of the pearl millet plant may range from 0.5 to 4 m, depending on the genotype and the grain can be nearly white, pale yellow, brown, grey, slate blue or purple. The early cultivars can mature within 60 to 95 days; the latest ones within 120 to 150 days (Thakur, 2004).

2.4 Production constraints

The crop is grown on the poorest soil and under harsh climatic conditions, demonstrating highest level of tolerance to drought and heat amongst the domesticated cereals (Budak *et al.,* 2003). The major constraints to the productivity of crop include lesser area under hybrids and prevalence of different biotic and abiotic stresses. Cultivation on marginal lands, unreliable rainfall tends to keep the use of inputs such as fertilizers to a minimum in this crop. Limited commercial demand depresses the incentive to use purchased inputs. Diseases are endemic to pearl millet in India. The four major diseases that cause economic losses to pearl millet are downy mildew (*Sclerospora graminicola*), ergot (*Claviceps fusiformis*), smut (*Tolysporium penicillariae*) and rust (*Puccinia substriata* var. indica) which develop depending on the weather conditions and genotype. Downy mildew is a recurring threat, threatening high-yielding varieties since the 1970s. Ergot and smut lower only grain yield, but downy mildew also kills plants. Chemical

control and resistance breeding have been employed in the management of downy mildew disease, but these methods have their own limitations and disadvantages. In view of the increasing severity of the downy mildew disease and evolution of new more virulent pathotypes, there is a need to develop a long-term strategy for DM resistance breeding in India (Thakur *et al.,* 2008).

2.5 *Sclerospora graminicola*: The causal organism of Downy mildew

Among various phytopathogens, the fungi have been historically and are currently the most important pathogens of crops causing far-reaching damages all over the world. As obligate biotrophs downy mildews are host-dependent. Co evolution with plant hosts over a long period has lead to divergent forms of pathogen adapted to different host taxa. The diploid vegetative stage of downy mildew pathogens contrasts with the haploid state of the true fungi (Fig 2.1 and Fig 2.2). During the sexual phase oospores are formed which are thick-walled and long-lived, and enable the pathogens to survive the crop-free, adverse periods (Ramalingam and Rajasab, 1981; Singh, 1995). Oospores are the primary inoculum source. The asexual phase occurs in periods of conducive weather conditions. Asexual reproduction is through the production of conidia or sporangia. Sporangia produce zoospores which are the infecting propagules, while conidia germinate directly. *Sclerospora graminicola* requires surface wetness for spore germination and infection along with high relative humidity (RH) for spore production. Thus, rainfall and high RH are critical weather factors for epidemics to develop. A minimum of 3 h at 25^0C and 95% RH is essential to initiate sporulation in *S. graminicola* (Singh *et al*., 1993). Most sporulation occurs when temperatures are around 21^0C and RH>95% (Payak, 1975; Shetty, 1987). Sporangia remain viable for 2.5–6 h depending on temperature, RH, wind speed (Shetty, 1987), and

under favorable conditions, they can be transported up to 3 km by wind. Seed transmission through oospores and mycelium has been reported (Shetty *et al*., 1978), but subsequent studies indicated that if the seed was dried to a moisture level of 12% the seedborne transmission could be eliminated (Williams, 1984). In *S. graminicola*, at least four distinct pathotypes have been established (Thakur *et al*., 1999). These pathotypes have evolved by host cultivar-directed selection under farmer's field conditions. Field screening using a combination of spreader rows and oospores infested plots has been used effectively to screen for resistance to the downy mildews of pearl millet at several locations in India (Thakur *et al*., 1992).

Considerable studies have been carried on the molecular identification of downy mildew isolates by various techniques like RAPD (Jogaiah *et al*., 2008), SCAR, ISSR (Sudisha *et al*., 2009), AFLP markers (Singru *et al*., 2003). Heterogeneity in terms of virulence and aggressiveness has been previously reported among the isolates (Thakur and Shetty, 1993). Biological pathotyping indicates diversity in the fungal pathogenic populations of *S. graminicola* in aspects such as host specificity, adaptation, and virulence (Thakur *et al*., 1992).

2.6 Disease resistance

Plants have different defense mechanisms to evade fungal invasion. Plants resist pathogen invasion by deploying various defense responses that are activated by two main branches of their immune system (Jones and Dangl, 2006). The first branch consists of transmembrane pattern recognition receptors (PRRs) that activate basal defense responses by recognizing extracellular pathogen-associated molecular patterns (PAMPs) common to many classes of microbes. Successful pathogens defeat this first line of defense with effectors that enhance their virulence. The second line of plant

defense, called ‘gene-for-gene’ resistance. This type of resistance is based on the presence of specific resistance (R) genes that mediate the recognition of race-specific effectors (*Avr* proteins) (Kim *et al*., 2005; Takken *et al*., 2006). These include oxidative burst, lignifications or specific enzyme activated cell wall reinforcement, production of secondary plant metabolites, hypersensitive cell death, and induction of systemic acquired resistance in distal plant organs and the transcriptional activation of numerous defense genes (Madhu *et al*., 2001).

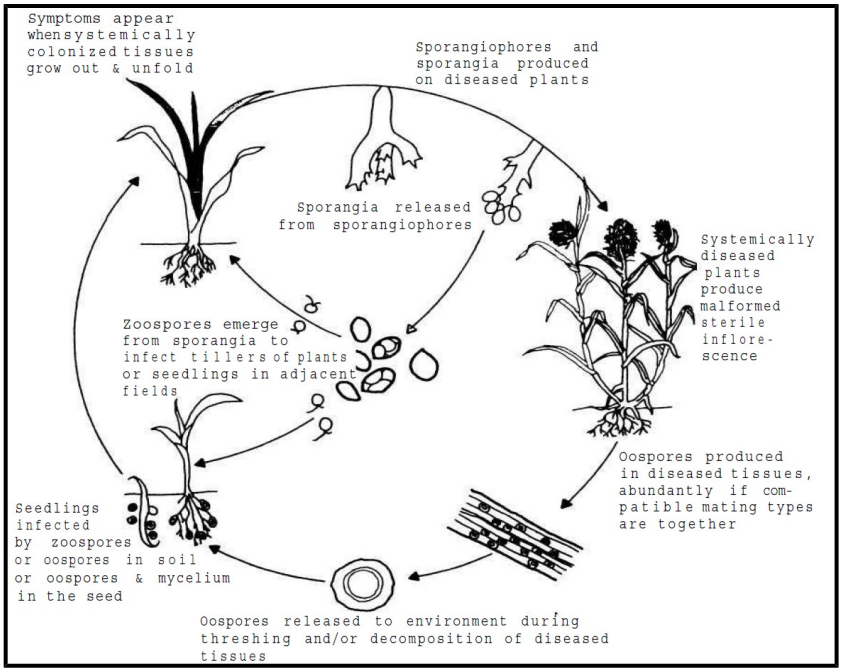

Fig 2.1 Life cycle of *Sclerospora graminicola*

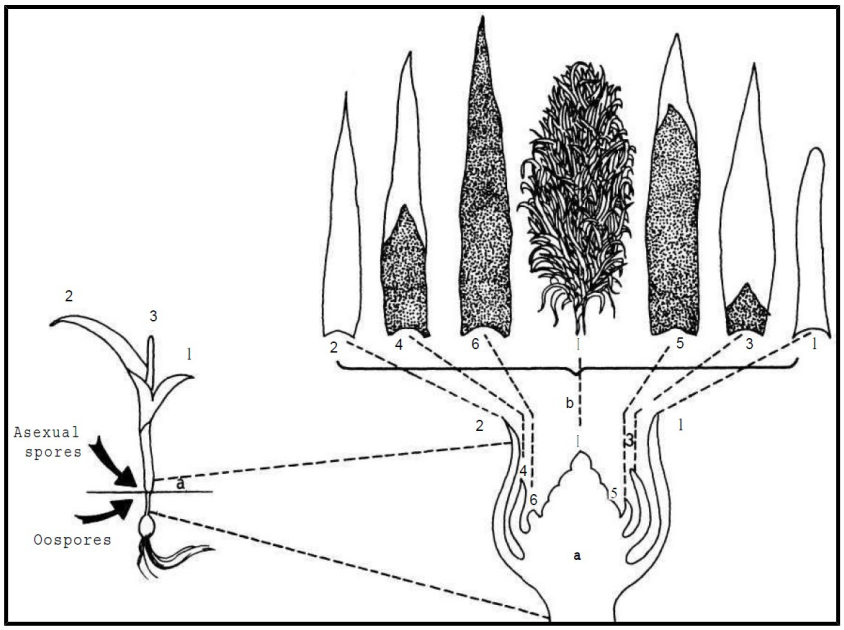

Fig 2.2 Progressive development of leaf symptoms in pearl millet colonized by *Sclerospora graminicola* (a) the pathogen colonizes the growing point of a seedling; (b) the tissues differentiated after colonization appear systematically diseased when the organs subsequently grow out and unfold.

2.6.1 Host-Pathogen interaction

Plants confronted with phytopathogens react to the challenge in a number of different ways. Development of defense responses in plants is complex and involves both structural and biochemical barriers (Paxton and Groth, 1994). Infection of plant tissues by a pathogen initiates a complex progression of morphological, biochemical, molecular and physiological interactions that culminate in visual symptoms associated with disease or disease resistance. The nature, timing and spatial coordination of the actions taken by either plant or pathogen are crucial in defining the result of any given interaction and thus the overall outcome of the infection. There are three kinds of host-pathogen interactions, namely, (i) non–host interaction which is responsible for the basic resistance in all plants, (ii) compatible interaction occurring between a susceptible host and its threatening

pathogen resulting in disease and, (iii) incompatible interaction that takes place between a resistant host and its pathogen that cannot cause disease (Singru *et al*., 2003).

2.6.2 Gene for gene concept and hypersensitive response

Two component systems explored the existing natural defense mechanisms in plants. The host-pathogen interaction in the pearl millet-DM system is expected to follow the general gene-for-gene concept (Flor, 1971) as is well known in other obligate systems, such as wheat-rusts, wheat-powdery mildew and lettuce-DM. This concept is based on major R-genes for resistance in host and complementary virulence genes in the pathogen. Resistance reactions are activated in plants harboring an R-gene and the corresponding *Avr* under the control of a strictly pathogen inducible promoter, upon pathogen attack. The gene-for-gene relationship envisions that dominant resistance genes interact with fungal avirulence genes, which usually results in a hypersensitive response (HR). Hence, biochemically, every resistance gene in the host could code for a receptor molecule that would directly or indirectly effectuate a HR after interaction with the product of an avirulence gene of the pathogen, the signal molecule, also called race-specific elicitor (Keen *et al,* 1982). Thus, three types of genes are involved in the HR response: resistance genes, regulator genes and defence genes (Van Loon *et al*., 2006). The HR involves rapid death of a few cells around the site of penetration, observable as a lesion (Tomiyama, 1982). The necrotic cells often serve as a reservoir of antimicrobial compounds, including phytoalexins that are synthesized in cells surrounding the lesion. The deposition of callose, lignin, hydroxyproline-rich glycoproteins and the accumulation of pathogenesis-related (PR) proteins such as β 1, 3-glucanases and chitinases also occurs in and around the lesion (De Wit, 1992).

Lignification occurs through a series of enzymatic steps, starting with a phenylalanine ammonia-lyase catalyzed reaction to produce lignin precursors, and terminating with a process that requires H_2O_2 and a cell wall-bound peroxidase (Olson and Varner, 1993).

2.6.3 Defense signaling

The plant cell is a stochastic environment of recognition receptors and the downstream signaling molecules, it should not be uncommon for crosstalk between resistance genes to occur. This is also economically sound for the plant because there would be a conservation of precious cellular and molecular resources during pathogen defence (Koornneef *et al*., 2008). Disease resistance in plants is associated with the activation of a wide variety of defense responses that serve to prevent pathogen infection. Several transduction pathways are activated following the initial recognition signals through a series of cytosolic and membrane-delimited pathways (Blumwald *et al*., 1998). The recognition of fungal elicitor by host plasma membrane receptors is considered to be the most important among signal events leading to activation of inducible defense mechanisms (Xing *et al*., 1997). The induced state can be achieved by increasing the production of a range of defense-related products such as defense induced signaling compounds, pathogenesis-related proteins, and phytoalexins (Deepak *et al*., 2007). The molecular basis for recognition of potential pathogen includes a multitude of plant resistance associated reactions that are initiated through the activation of host plasma membrane H^+ ATPase (Dixon *et al*., 1994), ion fluxes across the plant plasma membrane (Gelli *et al*., 1997). Oxidative burst is a phenomenon exhibited by living cells challenged by microbial pathogens/elicitors. Active oxygen species namely superoxide (O_2), hydroxyl radical (OH^+) and hydrogen peroxide (H_2O_2) are formed at the surface of cell

membrane (Mehdy, 1994). It stands temporarily between the earliest events such as the stimulation of ion fluxes across the plasma membrane and the later signals for the gene expression. It is also a part of central component in the integrated signaling systems, which amplify the response locally and at a distance. ROS have been suggested to be the first line of defense against pathogen invasion either by directly killing the pathogen or slowing down its ingress due to its rapidity of production and cytotoxicity (Geetha and Shetty, 2002).

2.6.4 Defense related genes and enzymes

Genes involved in the plant-pathogen interaction are known as ‘defense related genes’ that are regulated constitutively or by parasitism (Heath, 1991). Plant defense mechanisms operate through the activation of multiple defense related proteins. Promoter elements and transcription factors bind to the genes and regulate plant responses to pathogens. WRKY, homeodomain leucine zipper, ethylene responsive element binding protein (EREBP), F-box and Myb protein families are major transcription factor which play important role in defence response (Rushton and Somssich., 1998; Ulker and Somssich, 2004). RNA helicases had been reported to play an important role during development and stress responses in various organisms (Burg and Takken, 2009). Transcriptional activation of genes is a vital part of plant defence system against pathogens.

In fungal plant pathogenesis, enzymes play a crucial role through external and internal interactions to restrict the development of fungal pathogens. Enhanced biosynthesis and activity of some enzymes is one of the most important processes in plant defense and in some specific plant–fungal pathogen interactions the presence or activities of enzymes can be used as biochemical markers for the degree of resistance and/or susceptibility (Lebeda *et al*., 2000). The presence or

activities of enzymes can be used as biochemical markers for the degree of resistance or susceptibility (Raj *et al.,* 2006). The products of these enzymes are thought to act as elicitors for further induction of the enzymes and for the activation of other defense related biochemicals such as phytoalexins production and lignification. Table 2.1 shows the cereal resistance genes in response to various plant-pathogen interactions (Ayliffe and Lagudah, 2004).

Peroxidases are implicated to play key/multiple roles in plant-pathogen interaction. Pearl millet seedlings inoculated with downy mildew disease-causing pathogen *Sclerospora graminicola* shows differential expression of peroxidase activity. Involvement of defense related enzymes like lipoxygenase, phenylalanine ammonia lyase and β-1, 3-glucanase in pearl millet interaction is well studied in pearl millet downy mildew interaction (Babitha *et al*., 2006; Kini *et al*., 2000).

Pathogenesis related (PRs) proteins are expressed in plant fungal interaction which includes peroxidase, chitinase, thionine, thaumatin, glucanase and oxidase. Chandrashekhara *et al*., (2010) purified 5 kDa thionins from pearl millet which had anti-mildew activity tested on *S. graminicola*. The high level of transcript expression of thionins in resistant compared to susceptible cultivars was observed. Induction of thionins was also studied in barley leaf in response to the powdery mildew (Bohlmann *et al*. 1988).

Table 2.1 Cereal resistance genes involved in plant pathogen interaction

Species	**Gene**	**Protein**	**Pathogen**	**Disease**
Barley	*Mlo*	Mutant seven transmembrane	*Blumeria graminis*	Powdery mildew
	**Mla1*	NBS-LRR	*Blumeria graminis*	Powdery mildew
	**Mla6*	NBS-LRR	*Blumeria graminis*	Powdery mildew
	Rpg1	Protein kinase	*Puccinia*	Stem rust

			graminis	
Maize	*Rp1-D*	NBS-LRR	*Puccinia sorghi*	Leaf rust
	Rp3	NBS-LRR	*Puccinia sorghi*	Leaf rust
	Hm1	HC toxin reductase	*Cochliobolus carbonum*	Southern corn leaf blight
Rice	*Xa21*	Receptor kinase	*Xanthamonas oryzae*	Bacterial blight
	Xa1	NBS-LRR	*Xanthamonas oryzae*	Bacterial blight
	Xa26	Receptor kinase	*Xanthamonas oryzae*	Bacterial blight
	Pi-b	NBS-LRR	*Magnaporthe grisea*	Rice blast
	Pi-ta	NBS-LRR	*Magnaporthe grisea*	Rice blast
Wheat	*Lr21*	NBS-LRR	*Puccinia triticina*	Leaf rust
	Lr10	NBS-LRR	*Puccinia triticina*	Leaf rust
	Pm3	NBS-LRR	*Blumeria graminis*	Powdery mildew

* Other members of the *Mla* family not included in this table have also subsequently been shown to confer resistance to *Blumeria graminis*.

In plants, short chitin oligosaccharides and chitosan fragments are well-known elicitors that trigger defense gene expression, synthesis of antimicrobial compounds, and cell wall strengthening. Chitinases are structurally related to a class of xylanase inhibitors (Durand *et al*., 2005), which interfere with degradation of cell wall material. Huckelhoven, 2001 showed that chitinases hydrolyse the chitin of fungal cell walls and inhibit the growth of fungi in barley-powdery mildew interaction. Shivakumar *et al*., (2000) demonstrated the significance of ribonuclease in pearl millet–downy mildew interaction and its involvement of in systemic acquired resistance of pearl millet against the downy mildew pathogen. Sarosh *et al*., (2005) studied the induction of resistance in pearl millet against downy

mildew. The mRNA levels of genes for Pr-1a, beta 1, 3-glucanase, chitinase, peroxidase, lipoxygenase and chalcone synthase showed increase after inoculation with downy mildew pathogen.

Peroxidases participate in a variety of plant defense mechanisms in which H_2O_2 is often supplied by an oxidative burst, a common event in defense responses. Peroxidases are also implicated in hypersensitive response, lignin biosynthesis, ethylene production and suberization (Shivakumar, 2003). The association of increased peroxidase activity and the onset of systemic acquired resistance and hypersensitive response have been observed in a number of plant species, including cucumber, tobacco, melons, rice, wheat and lima beans (Hammerschmidt *et al*., 1982; Rasmussen *et al*., 1995; Young *et al*., 1995; Maffei *et al*., 2006).

Arun *et al*., (2010) estimated biochemical changes in susceptible and resistant cultivars of pearl millet on the basis of enzyme activities of peroxidase (POX), polyphenol oxidase (PPO), catalase (CAT) and IAA oxidase (IAAO) for understanding biochemical mechanism of disease resistance. Peroxidase (POX) activity was greatly increased in green ear affected with downy mildew of resistant pearl millet cultivar in comparison to the healthy as well as susceptible ones.

Shivakumar, (2003) carried out the differential expression of peroxidase isozymes in pearl millet seedlings inoculated with downy mildew disease-causing pathogen *Sclerospora graminicola*. Time-course study of peroxidase analyzed spectrophotometrically, showed increased activity at 8 and 16 h after inoculation in highly resistant cultivar (IP 18292) and highly susceptible cultivar (HB 3), respectively. On inoculation highly resistant, resistant and induced-resistant seedlings recorded increase in enzyme activity, whereas susceptible and highly susceptible seedlings recorded decrease in enzyme activity.

Swarbrick *et al*., (2008) carried out expression studies of rice Nipponbare roots infected with Stiga infection. Several genes encoding enzymes involved in defence-related secondary metabolism, including chalcone synthase and phenylalanine ammonia lyase (PAL), xylanase inhibitor, cytochrome P450 monoxygenases (P450s) were highly up-regulated.

Accumulation of flavonoids and isoflavonoids in response to pathogen attack and their importance as antibiotic phytoalexins is well established. Defense response induction of ABC transporters (Campbell *et al*., 2003) and their significance in conferring resistance to antifungal compound has been established (Boddu, 2006). The PHD fingers are present in most chromatin modification complexes (Slama and Geman, 2010) which have significance in plant defense. The F-Box Protein ACRE189/ACIF1 regulates cell death and defense responses activated during pathogen recognition in tobacco and tomato (Burg, 2008). Involvement of polyamine (Cowley and Walters, 2002), ethylene (Chen *et al*. 2003) and lignin accumulation in cereals (Bushnell, 2002) due to the infection of biotrophic pathogens have been described.

ROS inducers stimulate the induction of detoxification mechanisms, such as SOD and glutathione-S transferase and activation of other defence mechanisms in neighbouring cells (Shetty *et al*., 2008). Glutathione S-transferase plays a major role in secondary metabolite synthesis, mainly by regulating the key enzymes phenylalanine ammonium lyase (PAL) and chalcone synthase (CHS) (Gomez *et al*., 2004). Christensen *et al*., (1998) showed that a chalcone synthase was expressed in barley leaves in response to pathogen attack during the interaction between barley-*Blumeria graminis* f.sp. *hordei* (*Bgh*).

Caffeoyl-CoA O-methyltransferases (CCoAOMTs) is an important enzyme that participates in lignin biosynthesis especially in the

formation of cell wall ferulic esters of plants. Bhuiyan *et al*., (2009) studied the differential expression of PAL, CC0AOMT in wheat against powdery mildew invasion and played a pivotal role in cell wall reinforcement during the induced disease resistance response.

hsp 90 proteins (Sangster and Queitsch, 2005) and proteases (Xia, 2004) have frequently been found to be involved in or associated with induction of plant disease resistance. NBS-LRR R protein activity may also be subject to regulation by heat-shock proteins such as the hsp90 proteins (Belkhadir *et al*., 2004) thus establishing correlation between both in plant defense. Proteolytic pathway involves the small protein ubiquitin (Ub), which attaches to proteins destined for degradation; the resulting Ub-protein conjugates are then recognized and catabolised by the F-box proteins that act as recognition modules to specifically target their dedicated substrates for ubiquitylation. The substrate specificity is conferred by a C-terminal protein-interaction motif, often comprised of kelch repeats, leucine-rich repeats.

The Ca^{2+} ion is a second messenger in numerous plant signaling pathways, coupling extracellular stimuli to intracellular and whole-plant responses. The changes in Ca^{2+} concentration carry encrypted messages in the form of calcium signatures to calcium sensors where they are interpreted into appropriate biochemical and molecular responses for a specific physiological stimulus (Snedden and Fromm, 2001; Yang and Poovaiah, 2003). Calmodulin (CaM) is the best characterized Ca^{2+}binding protein and its role in Ca^{2+} signal transduction has been widely investigated (Reddy, 2001; Snedden and Fromm, 2001). The importance of nuclear Ca^{2+} in signaling processes is underlined by the existence of Ca^{2+} effectors in the plant nucleus, including calmodulin (CaM), CaM-binding protein, CDPKs and Ca^{2+}-CaM-regulated protein phosphatises (Vadassery and Oelmüller, 2009). G proteins act as molecular signal transducers whose active or inactive

states depend on the binding of GTP or GDP and GTP/GDP cycle acts as a molecular switch for signal transduction. Serine kinase has been shown to act as a positive regulator in infection induced cell death signaling (Chinchilla *et al*., 2007). Phosphorylated derivatives of PtdIns, known as phosphoinositides, play a key role in the membrane recruitment and or activation of proteins. Phosphoinositide 3-kinases play a role in signal transduction, membrane trafficking, cytoskeletal regulation (Gillooly, 2001). Mitogen-activated protein kinase (MAPK)-cascades play key roles in the change of the phosphorylation pattern (Miles and Ellis, 2000). Chae *et al*., (2009) showed that Pmk1 mitogen-activated protein (MAP) kinase is essential for appressorium formation and infectious growth in the rice blast fungus *Magnaporthe grisea* interaction. Plant RLKs receptor-like kinase genes are serine/threonine kinases that represent a novel signaling innovation unique to plants. Genes encoding RLKs are involved in a range of environmental and developmental responses, including recognition of pathogens groups bacterial (*Xa21),* fungal *(FLS2),* and viral pathogens *(EFR)* elicited the largest proportion of differentially expressed RLK genes (60%) in *Arabidopsis* RLK genes.

Differential expression of genes in response to the interaction between the pathogen *Fusarium graminearum* and its host, *Hordeum vulgare* was observed by Geddes (2008). Arabidopsis Snf1-related protein kinases (SnRKs) are implicated in pleiotropic regulation of stress responses through their interaction with the kinase inhibitor WD protein (Farraas *et al*., 2001). Tomato RAV transcription factor is a pivotal modulator involved in the AP2/EREBP-mediated defense pathway (Li *et al*., 2011). The glycogen synthase kinase 3 (GSK3)/SHAGGY-like kinases (GSKs) are non receptor serine/threonine protein kinases that are involved in a variety of biological processes (Yoo *et al*., 2006).

Swarbrick *et al*., (2008), profiled the changes in gene expression in susceptible (IAC 165) and resistant (Nipponbare) rice cultivars using rice whole-genome microarrays. The resistance reaction was characterized in by up-regulation of defence genes, including pathogenesis-related proteins, pleiotropic drug resistance ABC transporters, genes involved in phenylpropanoid metabolism and WRKY transcription factors. The susceptible interaction was characterized by large-scale down-regulation of gene expression, particularly within the functional categories plant growth regulator signaling and metabolism, biogenesis of cellular components and cell division.

2.7 Transcriptome sequencing

The field of transcriptomics focuses on studies, where experiments are designed to monitor and manipulate the dynamics of gene expression events that occur during both resistance/tolerance and susceptibility to particular stresses. The Genome Sequencer FLX System is a powerful platform for transcriptome sequencing and quick, unbiased transcriptome surveys of unknown genomes can be obtained in order to assess new gene functions, novel-transcript identification, polymorphisms for genotyping (SSR, SNP discovery), expression analysis alternative splice variants, and other sequence motifs at unprecedented speed (Schuster, 2008). With the advent of next-generation sequencing, transcriptomic data for many species became available (Maria *et al*., 2011).

2.7.1 Genome Sequencer FLX System (GS FLX)

The Genome Sequencer FLX System (GS FLX), powered by 454 Technologies and commercialized by Roche 454 Sequencing, is the first commercial next-generation DNA sequencing technology featuring a

unique mix of long reads, exceptional accuracy, and ultra-high throughput (Imelfort and Edwards, 2009). 454 pyrosequencing provides sufficient length of sequence information to allow accurate annotation without the need for a reference genome sequence (Margulies *et al*., 2005). The 454 technology is well suited to EST sequencing and provides an unbiased representation of all regions of a transcript independent of length or expression level. 454 Roche has provided unprecedented opportunities for high-throughput functional genomic research (Morozova and Marra, 2008). It circumvents the cloning requirement by taking advantage of a highly efficient *in vitro* DNA amplification method known as emulsion PCR and the sequence of DNA template is determined from a "pyrogram". The Roche 454's FLX technology is capable of producing reads 200–300 bp in length and >100 Mb of sequence data from a single 7-h plate run. The new improved 'GS FLX Titanium' technology allows for reads of >400 bp and a total of >500 Mb of sequence data per run at 99.5% accuracy per run. It also promises to eliminate the problem of encountering long homopolymer runs such as poly-T stretches and generate more data per run than Roche 454, but at the expense of shorter reads of < 40 bp (Coram *et al*., 2008).

2.7.2 Chemistry

DNA sequencing-by-synthesis (SBS) technology, which employes a polymerase or ligase enzyme as its core biochemistry, has been incorporated in 454 Pyrosequencing (Carl *et al.,* 2009). The GS FLX Titanium Chemistry employs a robust set of ten decamer Multiplex Identifier (MID) sequences to facilitate library multiplexing in the 454 sequencing system. Pyrosequencing technology relies upon enzyme cascades and CCD luminescence detection capabilities to measure the release of inorganic pyrophosphate with every nucleotide incorporation

capture beads that contain, on average, one single-stranded template, which is amplified to millions of copies in an oil emulsion PCR (emPCR). The beads are then distributed on a solid-phase sequencing substrate (a PicoTiterPlate™) with 1.6 million wells that can each hold a bead and additional reagents, including polymerase, luciferase, and ATP sulfurylase.

Microfluidics, cycle each of the four nucleotide triphosphates over the PicoTiterPlate™, and incorporation of a nucleotide releases pyrophosphate, the substrate for a luminescence reaction, which is recorded with a cooled CCD camera. The record of intensity of each flow of a nucleotide is a flowgram, analogous to a chromatogram that reports the order of A, C, G and T residues from a DNA sequencing template. Flowgram values correspond to the homopolymer length for that base. The outline of GS FLX workflow is presented in Fig. 2.3.

Table 2.2 Approach and Length distribution (bp) of reads from the Sanger, 454, and hybrid assemblies.

Technology	Approach	Read Length	bp per run	Company name
Automated Sanger sequencer	Synthesis in the presence of dye terminators	Up to 900bp	96 kb	Applied Biosystems
454/Roche FLX	Pyrosequencing on solid support	200-300 bp	80-120Mb	Roche Applied Science
Illumina/Solexa	Sequencing by synthesis with reversible terminators	30-40 bp	1 Gb	Illumina , Inc.
ABI/SOLID	Massively parallel sequencing by ligation	35 bp	1-3 Gb	Applied Biosystems

2.7.3 Software

The software identifies as ambiguous flow cycles in which no flowgram value was greater than 0.5. If 5% or more of the flow cycles for a read are ambiguous, the read is removed. The 454 pyrosequencer performs data analysis without the need for enterprise scale IT solutions with the included easy-to-use software tools - GS *De Novo* Assembler, GS Reference Mapper, and GS Amplicon Variant Analyzer. Straightforward interpretation of data means faster discovery of biologically meaningful results.

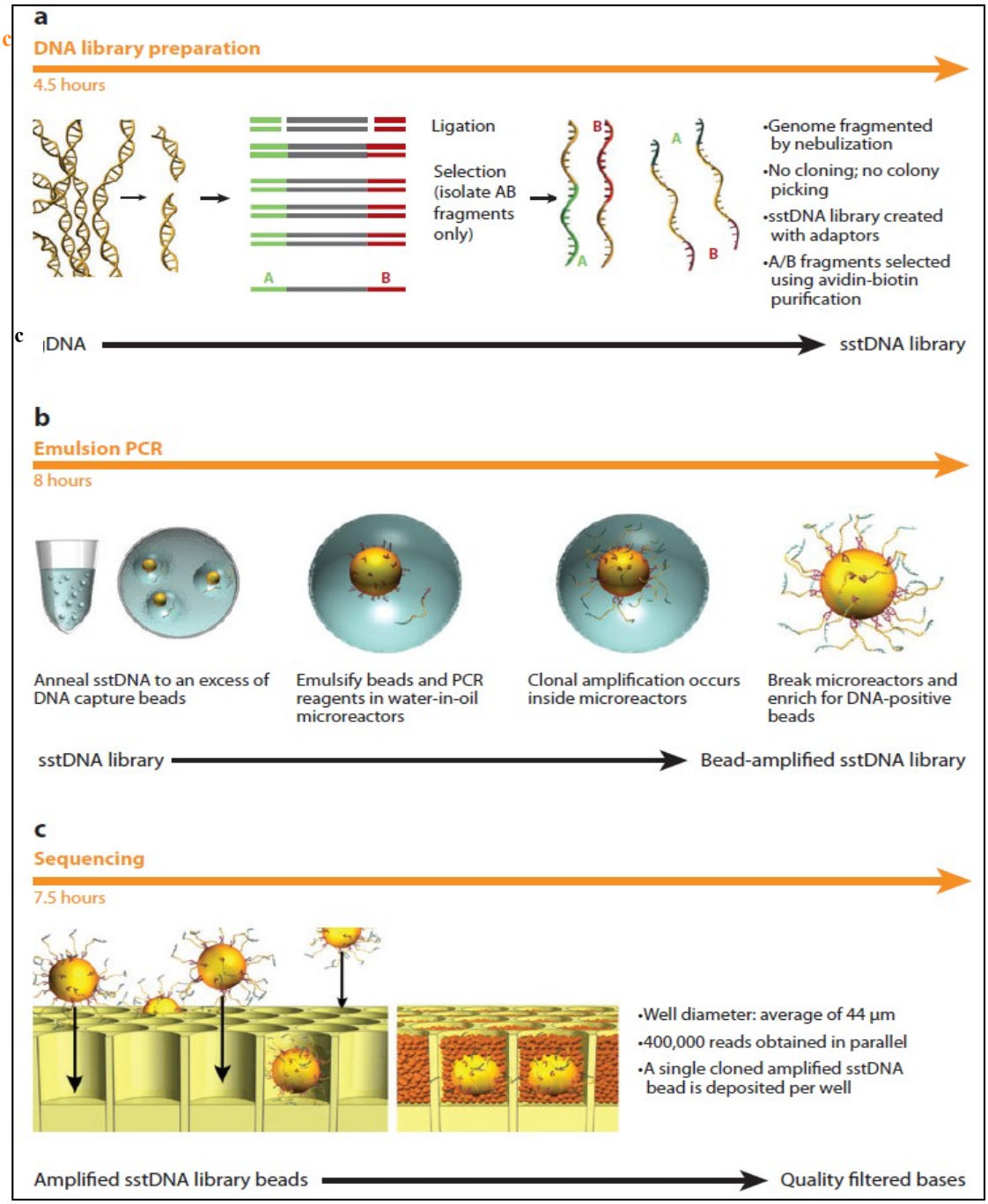

Fig 2.3 Outline of the 454 GS FLX™ DNA Sequencer workflow showing

Library Construction, Emulsion PCR and Sequencing

2.7.4 Recompense

Roche 454 sequencing has clear advantages over microarrays for transcriptomics research, because it is an open system that can potentially capture all the transcripts in a given sample and can also accurately measure gene expression by counting the number of identical transcripts detected (Coram *et al*., 2008). Since the nucleotide triphosphates are flowed one at a time, substitutions are less likely than with traditional methods. Sequencing-by-synthesis approach is capable of producing much deeper coverage of the transcriptome than Serial Analysis of Gene Expression (SAGE) and Massively Parallel Sequencing by Synthesis (MPSS) (Brenner *et al*., 2000) and promises to combine the high-throughput SAGE and MPSS with the accuracy of EST sequencing. Coram *et al.,* (2008) considered Roche 454 technology as the best option for future progress because of its longer sequence reads that can be more easily annotated, as well as its unbiased potential for covering the entire wheat transcriptome.

Chum *et al.,* (2011) using the 454 GS 20 sequencing system, sequenced cDNAs pooled from sporeless (FB) and spore-bearing (FBS) fruiting bodies and investigated the molecular mechanism of the fruiting body development and sporulation in the cap of the Shiitake mushroom, *Lentinula edodes*. A large number of *L. edodes* cDNA sequences (>7000 in this study) were generated quickly and efficiently, paving the way for the establishment of an expressed gene catalog for this mushroom. Gene Ontology was used to categorize the contigs to form the catalog of genes expressed at the stage of the mature fruiting body and also assigned the contigs into the KEGG pathways to depict the detailed metabolic pathways and processes occurring in the mature fruiting body.

Bai *et al*., (2011) obtained 58,673 high quality ash sequences from pooled phloem samples of green, white, black, blue (highly

susceptible) and Manchurian ash (resistant) to *A. Planipennis.* Various metabolic pathways that are of high interest with respect to ash resistance to *A. Planipennis* were found. Expression analysis of early regulators potentially involved in plant defense (i.e. transcription factors, calcium dependent protein kinases and a lipoxygenase 3 revealed higher mRNA levels in resistant ash compared to susceptible ash species. SNPs and SSRs were predicted, out of which seven microsatellite loci showed polymorphism between different ash species.

Blanca *et al*., (2011) generated *Cucurbita pepo* EST sequences, using 454 GS FLX Titanium technology from normalized cDNA libraries (root, leaves, and flower tissue) prepared using two varieties with contrasting phenotypes for plant, flowering and fruit traits, representing the two *C. pepo* subspecies: subsp. pepo cv. Zucchini and subsp. ovifera cv Scallop. Genes potentially encoding proteins involved in pathogen resistance, flowering, fruit quality and root traits were identified.

Der *et al*., (2011) exploited Roche 454 GS-FLX Titanium pyrosequencing platform for sequencing the gametophyte transcriptome of bracken fern (*Pteridium aquilinum*) in order to develop genomic resources for evolutionary studies. A total assembly size of 30.8 Mbp with average read-depth coverage of 7.0X was produced. A comparative genomics approach revealed a substantial proportion of genes expressed in bracken gametophytes to be shared across the genomes of *Arabidopsis, Selaginella* and *Physcomitrella*, and documented a substantial number of potentially novel fern genes.

Moe *et al*., (2011) employed 454 sequencing technology for transcriptome sequencing of mungbean (*Vigna radiata*). Mungbean's ability to fix nitrogen, early maturity and relatively good drought resistance were explored. The transcriptomic data was used for

implementing a functional annotation and development of genetic markers, which can be put to use for mungbean improvement programs.

Narina *et al*., (2011) investigated gene expression, using Roche 454 technology, from young leaves one susceptible (TDa 95-0310) and two resistant yam genotypes (TDa 87-01091, TDa 95-0328) challenged with the fungus, Anthracnose (*Colletotrichum gloeosporioides*) which is a major limiting factor in the production of yam (Dioscorea spp.). Mining for SSRs in the ESTs revealed 1702 unique sequences containing SSRs and 1705 SSR markers were designed using those sequences. ESTs were annotated using MapMan Mercator tool and Blast2GO search tools and characterized the transcriptomes for differential gene expression, transcripts for disease resistance in the resistant and susceptible genotypes.

Swarbreck *et al.,* (2011) carried out 454 transcriptome sequencing of leaf and root samples of slender wild oat, *Avena barbata* from the plants grown on natural soil and under varying rainfall patterns. Differential expression of five root-specific genes under three water levels and two developmental stages was evaluated. Numerous candidate polymorphic markers in the data set, providing possibilities for linking the genomic and the existing genetic information for *A. barbata* were identified. Through a combination of Sanger and 454-based sequencing technologies, a large set of transcribed sequences for *A. Barbata* was generated.

Sato *et al*., (2010), subjected cDNAs derived from leaf and callus tissues of *Jatropha curcas* to pyrosequencing, in parallel with genome sequencing by the conventional Sanger method. Microsatellite markers have been developed using the sequence information, and polymorphism among various *J. curcas* varieties genes related to flowering, disease resistance, transcription factors were identified.

Genes involved in synthesis of phorbol ester, triacylglycerols, and ribosome-inactivating protein- curcin biosynthesis were recognized.

Carolyn *et al*., (2010) characterized two genes involved in the fatty acid biosynthesis pathway namely, fatty acid desaturase (FAD) 2 and fatty acid elongase (FAE) 1 by using 454 sequencing. Sequencing revealed the unexpected complexity in *C. sativa* genome and fatty acid synthesis pathway. These genes will allow the future targeted manipulations of oil composition of this emerging biofuel crop.

Roland *et al.,* (2010), normalized cDNA collections from stems and leaves from drought stressed sweet potato SSR motifs were identified with the SSR locator on the basis of this gene index have identified 1,661 gene-based microsatellite sequences, of which 223 were selected for testing and 195 were successfully amplified in a test panel of six hexaploid (*Ipomea batatas*) and two diploid (*Ipomea trifida*) accessions.

Yang *et al.,* (2010) showed that one run of GS FLX generates sufficient genomic information for adequate *de novo* assembly of a large number of transcripts in a wild rice species, *Oryza longistaminata* using cDNA library from roots adapted to low nitrogen conditions. Comparison with ESTs derived from cultivated rice collections revealed expressed genes across different plant species suggesting different metabolic activity in *O. longistaminata* roots in comparison to *O. sativa* roots.

Riggins *et al*., (2010) sequenced the transcriptome of waterhemp for identifying target-site genes involved in herbicide resistance using Roche GS-FLX 454 pyrosequencing technology and identified important herbicide target-site genes in waterhemp, [4-hydroxyphenylpyruvate dioxygenase (HPPD) and glutamine synthetase], where resistance has not yet been reported in any plant.

Isabel *et al.,* (2010), using 454 GS-FLX Titanium pyrosequencing, identified expressed sequence tags (ESTs), assembled and annotated the transcripts encoding all known sanguinarine biosynthetic enzymes in fungal elicitated opium poppy cell cultures.

Dassanayake *et al*., (2009) presented the first transcriptome analysis of mangroves *Rhizophora mangle* (Rhizophoraceae) and *Heritiera littoralis* (Malvaceae) as ecologically important extremophiles employing markedly different physiological and life-history strategies for survival and dominance in extreme environment. The sequences substantially differed from the model plants *Arabidopsis* and *Populus*.

Barakat *et al.,* (2009) identified genes involved in resistance to *Castanea parasitica* by sequencing the transcriptome from fungal infected and healthy stem tissues collected from blight-sensitive American chestnut (*C. dentata*) and blight-resistant Chinese chestnut (*C. mollissima*) trees using ultra high throughput pyrosequencing. *In silico* expression analyses showed that many of the stress response unigenes were expressed more in canker tissues versus healthy stem tissues in both American and Chinese chestnut. Comparative analysis also identified genes belonging to different pathways of plant defense against biotic stresses that are differentially expressed in either American or Chinese chestnut canker tissues. The identification of many defense related genes differentially expressed in canker vs. healthy stem in chestnuts provided many new candidate genes for developing resistance to the chestnut blight and for studying pathways involved in responses of trees to necrotrophic pathogens.

Julio *et al.,* (2009) explored the transcriptional diversity in an ancient maize landrace and identified transcripts corresponding to 34% of public maize ESTs databases suggesting the molecular and functional diversity contained in the vast native landraces. The study

put forward a valuable approach to characterize the functional diversity of maize for future agricultural and evolutionary studies.

Alagna *et al.,* (2009) carried out sequencing of *Olea europaea* fruit cDNA collections namely *Coratina*, a widely cultivated variety characterized by a very high phenolic content and *Tendellone*, an oleuropein-lacking natural variant has provided large scale information about the structure and putative function of gene transcripts accumulated during fruit development. 454 pyrosequencing was used to enrich the very few sequence data currently available for the species and to identify genes involved in expression of fruit quality traits.

Sato *et al*., (2009) determined all the gene products involved in wood degradation performed massively parallel pyrosequencing on an expression library from the white rot fungus *Phanerochaete chrysosporium* grown in shallow stationary cultures with red oak as the carbon source and revealed new transcripts that encode extracellular proteins with no known function.

Carlos *et al.,* (2008) analyzed the gene expression in chickpea roots in response to drought, from drought-stressed and non-stressed roots with a combination of high-throughput 454 sequencing and SuperSAGE. The transcriptome of chickpea roots which represented most comprehensive analysis of the drought-response transcriptome of chickpea.

Pandey *et al*., (2008) characterized *Nicotiana attenuata's* smRNA transcriptome before and after insect-specific elicitation in wild-type (WT) and RdR1-silenced (irRdR1) plants. The targets of *N. attenuata* smRNAs in the genes related to phytohormone signaling known to mediate resistance responses were predicted.

Moore *et al*., (2006) carried out pyrosequencing of whole plastid genomes of the basal eudicot angiosperms *Nandina domestica*

(Berberidaceae) and *Platanus occidentalis* (Platanaceae). Nandina and Platanus which exhibited overall error rates of 0.043% and 0.031%, respectively, in the consensus sequence.

As the information available on pearl millet is scarce, the references cited here are from other plant species.

2.7.5 Bioinformatics Analysis

2.7.5.1 Functional Annotation: Blast2GO

Blast2GO (B2G) is a research tool designed with the main purpose of enabling Gene Ontology (GO) based data mining on sequence data for which no GO annotation is yet available. B2G joints in one application GO annotation based on similarity searches with statistical analysis and highlighted visualization on directed acyclic graphs. This tool offers a suitable platform for functional genomics research in non-model species. It is a user-friendly, easy to distribute and low maintenance tool. It allows monitoring and interaction at different steps of the analysis, and emphasizes visualization as an important component of knowledge acquisition. Functional annotation is a prerequisite to better understand transcriptomic data, especially of non-model systems (Conesa *et al*., 2005).

B2G has been design to (1) allow automatic and high throughput sequence annotation and (2) integrate functionality for annotation-based data mining. Briefly, B2G uses BLAST (Altschul *et al*., 1990) to find homologs to fasta formatted input sequences. The program extracts GO terms to each obtained hit by mapping to existent annotation associations. An annotation rule finally assigns GO terms to the query sequence. Blast expectation values (E-value) and hit number thresholds are provided to retrieve significant results. In transcriptome analysis Blastx is done as it compares translational products of the

nucleotide query sequence to a protein database. Translated BLAST services are useful when trying to find homologous proteins to a nucleotide coding region. Because blastx translates the query sequence in all six reading frames and provides combined significance statistics for hits to different frames, it is particularly useful when the reading frame of the query sequence is unknown or it contains errors that may lead to frame shifts or other coding errors. Thus blastx is often the first analysis performed with a newly determined nucleotide sequence and is used extensively in analyzing EST sequences. This search is more sensitive than nucleotide blast since the comparison is performed at the protein level. The GO facilitates functional characterization of genes, transcripts and proteins of any organisms with respect to cellular component, biological process and molecular function in a species independent manner as reported in several other studies. It is an attractive tool for research environments where genetic and/or computational resources are limited and where much work is still done in an explorative fashion. In a GO annotation, Evidence code indicates that the result from physical characterization of a gene or gene product has supported the association of a GO term.

2.7.5.2 Sequence cleaning: Repbase and EGassembler

Repbase Update is a comprehensive database of repetitive elements from diverse eukaryotic organisms. Sequences from Repbase Update are used to screen and annotate repetitive elements using programs such as Censor and RepeatMasker (Jurka *et al*., 2005).

EGassembler is a web server, which provides an automated as well as a user-customized analysis tool for cleaning, repeat masking, vector trimming, organelle masking, clustering and assembling of ESTs and genomic fragments. The web server is publicly available and provides the community a unique all-in-one online application web

service for large-scale ESTs and genomic DNA clustering and assembling. A significantly large volume of data can be processed in a short period of time. The results can be used to functionally annotate genes, to facilitate splice alignment analysis, to link the transcripts to genetic and physical maps, design microarray chips, to perform transcriptome analysis and to map to KEGG metabolic pathways (Masoudi, 2006).

2.7.5.3 Assembly program: CAP3

CAP3 is a sequence assembly program. It has a capability to clip 5’ and 3’ low-quality rboegions of reads. It uses base quality values in computation of overlaps between reads, construction of multiple sequence alignments of reads, and generation of consensus sequences. The program also uses forward–reverse constraints to correct assembly errors and link contigs (Huang and Madan, 1999).

2.7.5.4 Pathway database: KEGG

KEGG (Kyoto Encyclopedia of Genes and Genomes) is a knowledge base for systematic analysis of gene functions, linking genomic information with higher order functional information. The genomic information is stored in the GENES database, which is a collection of gene catalogs for all the completely sequenced genomes and some partial genomes with up-to-date annotation of gene functions. The higher order functional information is stored in the PATHWAY database, which contains graphical representations of cellular processes, such as metabolism, membrane transport, signal transduction and cell cycle (Kanehisa and Goto, 2000).

III. MATERIAL AND METHODS

Laboratory analysis was carried out at Centre of Excellence in Biotechnology, Dept. of Biochemistry, B. A. College of Agriculture and Dept. of Animal Biotechnology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand.

3.1 Materials

3.1.1 Chemicals, Buffers, Reagents

All the chemicals used for molecular and biochemical work during the course of study were of molecular and analytical grade obtained from standard manufacturers, Roche, Sigma, Invitrogen, Amresco, Merck through local dealer.

3.1.2 Glassware and plasticware

Properly cleaned and neutral glassware (Borosil grade) were used. The glasswares were sterilized in oven before use. Plasticware used for thermocycler and sequencing work were compatible with molecular biology work. All the plasticwares like micropipette tips, PCR tubes, centrifuge tubes and eppendorf tubes were chloroform treated and autoclaved before use.

3.1.3 Experimental material

The genotypes for the present study were procured from the Main Pearl Millet Research Station, Junagadh Agricultural University, Jamnagar, Gujarat.

Table 3.1 List of genotypes

Sr. No.	Genotypes
1	Resistant genotype: J-2290

2	Susceptible genotype: 7042 S

3.1.4 Treatments

1. J2290 Inoculated/ Stressed: Seedlings inoculated with Downy mildew pathogen inoculum
2. J2290 Control: Seedlings inoculated with sterile distilled water
3. 7042S Inoculated/ Stressed: Seedlings inoculated with Downy mildew pathogen inoculum
4. 7042S Control: Seedlings inoculated with sterile distilled water

3.1.5 Equipments and instruments

Agilent 2100 Bioanalyser, Agilent Technologies, USA

Gel Documentation machine: BioRad, USA

Ice flaking Machine: Icetronic, South Africa

Microwave oven: LG India Ltd., India

NanoDrop® ND-1000, Nanodrop Technologies, INC. Wilmington, USA; full spectrum U.V-Vis spectrophotometer

Hot air oven (Model-NV 858/859, Nova Instruments Pvt. Ltd., Ahmedabad, India

pH meter: Thermo Orion, Model 420, USA

Refrigerated Centrifuge: Sigma, USA

Thermal cycler: Gene Amp® PCR System 9700, Applied Biosystems, USA

Water bath with shaker: Nova, Cintex

Weighing Balance: BP 210 D, Sartorius, Germany

Leica Microscope: Leica DM 2500, Germany

TissueLyser, Quiagen, USA

AllegraTM 25 R centrifuge,

Deep Freezer (-200C), Vestfrost, UK

454 GS FLX Instrument, Roche, USA

TBS 380 Fluorometer, Promega, USA

3.2 Methods

3.2.1 Pathogen inoculation procedure

3.2.1.1 Seedling germination

The pearl millet seeds were surface sterilized in 0.1% sodium hypochlorite solution for ten minutes followed by three washes with sterile distilled water. The seeds were kept for germination on moist filter paper, 25 seeds/petri plate in triplicate under aseptic conditions in controlled-environment at 25 ± 2 ^{0}C in dark for two-days (ISTA, 1999). The two-day-old grown/two days after germination (DAG) seedlings were used for further inoculation.

3.2.1.2 Inoculum preparation

Infected leaves of pearl millet were collected in the evening from the susceptible genotype maintained in the polyhouse. The leaves were washed with tap water to remove the spores and fungal remnants present on the surface of leaves. Moist chamber was prepared by placing water soaked whatman filter paper in a petri plate, excess water was removed. The leaves were cut into ~2 inch length and placed in moist chamber on their upper side down. The lids were covered after placing the leaves. The plates were kept in cool place over night, covering the plates with a plastic tray. The sporangia were harvested next day early morning from the leaves using distilled water and a paint brush using minimum sterile distilled water to keep the spore suspension concentrated. After harvesting the spores, the suspension was kept in a dark place for 10-15 minutes for the zoospores release. The release of zoospores was checked under microscope by placing a drop of suspension on the glass slide and observed under microscope. The inoculum for infection was prepared by adjusting the zoospores count to 4x10^{4}/ml with sterile distilled water as read using a haemocytometer (Safeulla, 1976).

3.2.1.3 Inoculation of the two day old grown seedlings

The 2-day-old seedlings were root dipped inoculated with the inoculum suspension and the one with water served as control (Safeulla, 1976). Care was taken not to disturb the seedlings. The inoculated seedlings were incubated in a moist, dark chamber. The seedlings were observed time to time for hypersensitive response. The seedlings were removed after 36 hours from the zoospore suspension. The seedlings showing HR were cut into pieces and stored in RNA later at -80^{0}C for RNA isolation and peroxidase enzyme was extracted immediately. Peroxidase activity of the 36 hours post inoculation (hpi) seedlings was measured.

3.2.2 Determination of Peroxidase activity

3.2.2.1 Enzyme extraction

Extract for determination of peroxidase was prepared from 150mg seedlings homogenized within a pre-chilled mortar and pestle in icebox in 2 ml of extraction buffer, containing 50 mM sodium phosphate buffer (pH 7.2) with the addition of 1% polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 15 minutes and the supernatant was used for the assay (Bhatnagar *et al*., 2007). Protein content was estimated by the Folin Lowry method (Lowry *et al*., 1951).

3.2.2.2 Enzyme activity

The reaction mixture contained 2.99 ml of 0.03% H_2O_2 (substrate) in 0.1M phosphate buffer (pH 6.0), 1% orthodianisidine dye (freshly prepared, dissolved in methanol). The reaction was initiated by the addition of 10µl of enzyme extract. The change in color of oxidized dye was read at 460 nm upto one min at the interval of 15 sec. Blank

was run without the addition of substrate. The enzyme activity was expressed as change in O.D./min/g fresh tissue (Guibault, 1976).

3.2.3 Transcriptome analysis

3.2.3.1 Library preparation

3.2.3.1.1 Total RNA isolation

Total RNA was isolated from 300 mg of stored seedling tissues from resistant inoculated, resistant control, susceptible inoculated and susceptible control using TRIzol reagent (Invitrogen) following manufactures instruction. 1µl RNA sample was loaded into the well of Nanodrop spectrophotometer and the concentration of RNA and absorbance at 260 nm and 280 nm were measured.

3.2.3.1.2 mRNA isolation from total RNA

mRNA isolation from total RNA was carried out by using mRNA isolation kit, Roche. Procedure was followed according to manufacturer's instructions. The quality of mRNA was checked by running 1µl of each sample on RNA 6000 Pico Chip on the Agilent 2100 Bioanalyser.

3.2.3.1.3 Fragmentation of mRNA

mRNA was fragmented using RNA fragmentation solution by placing at 70 ° C for 10 minutes in thermocycler, immediately placing on ice for 2 min. The quality of fragmented mRNA was checked by running the sample on RNA 6000 Pico Chip on the Agilent 2100 Bioanalyser (Agilent Technologies, USA) as per the manufacturer's protocol.

3.2.3.1.4 Double stranded cDNA synthesis

cDNA synthesis was carried out by using cDNA Rapid Library Preparation kit, Roche. Primer random was added to the fragmented RNA and incubated at 70^0 C for 10 min followed by placing on ice for 2

min. First strand cDNA was synthesized using AMV reverse transcriptase and incubating at 25^0 C for 10 min 42^0 C for 60 min. Second strand cDNA was synthesized using T4 DNA Polymerase.

3.2.3.1.5 Double-stranded cDNA purification

The double stranded DNA was purified using AMPure beads followed by ethanol washes and a finally dissolving in Tris-HCl.

3.2.3.1.6 Fragment end repair

Fragment end repair was carried out by using End repair mix containing T4 Polymerase and Taq Polymerase.

3.2.3.1.7 Adaptor Ligation

After fragment end repair, RL Multiplex Identifier (MID) adaptors 5, 6, 7, 8 (Table 3.2) were ligated to the four samples by employing RL Ligase followed by incubation at 25° C for 10 min.

Table 3.2 Ten-base Extended Multiplex Identifier (MID) set sequences

Sample name	Mid adapter	Sequence
Resistant inoculated	MID-5 ATCAGACACG	ATCAGACACG
Resistant control	MID-6 ATATCGCGAG	ATATCGCGAG
Susceptible inoculated	MID-7 CGTGTCTCTA	CGTGTCTCTA
Susceptible control	MID-8 CTCGCGTGTC	CTCGCGTGTC

3.2.3.1.8 Small fragment removal

Small fragments were removed by treating the samples with sizing solution followed by ethanol washes. Finally TE buffer was added followed by further quantification.

3.2.3.1.9 Library quantification

Each of the four libraries was quantified using the TBS 380 Fluorometer. Standard curve was generated and library sample

concentration was determined using Rapid Library Quantification Calculator. Library quality and quantity assessment was done by running 1µl of single stranded DNA library on an Agilent 2100 DNA High Sensitivity chip on Agilent 2100 Bioanalyzer.

The library stock concentration in molecules/ µl was calculated as follows:

$$\text{Molecules/µl} = \frac{\text{(Sample conc.; ng/µl) X } (6.022 \text{ X } 10^{23})}{(328.3 \text{ X } 10^{9}) \text{ X (avg. fragment length)}}$$

After determining the concentration of cDNA molecules the libraries were diluted accordingly to get 1E7 molecules/µl and stored at -15^{0} C to -25^{0} C.

Table 3.3 Library dilution for emPCR

Sample name	Library dilution(1E7 molecules/µl) in TE
Resistant inoculated	28.5µl sample into 471.5 µl TE buffer
Resistant control	34.0µl sample into 466.0 µl TE buffer
Susceptible inoculated	50.0µl sample into 450.0 µl TE buffer
Susceptible control	2.1µl sample into 497.9 µl TE buffer

3.2.3.2 Emulsion based clonal amplification

The emulsion PCR (emPCR) was performed for *in vitro* amplification of the single stranded library of quantitated DNA fragments to generate library of clonally amplified, bead-immobilized, single-stranded DNA fragments representative of the entire transcriptome by using GS FLX Titanium SV emPCR emPCR Kit (Lib-L) and GS FLX Titanium LV emPCR Kit (Lib-L), Roche. To estimate the correct volume of diluted DNA library (1 X 10^{6} molecules/ µl) to be added to each tube of DNA capture beads, which would give best sequencing results, first, the library titration was performed using small volume emulsion (SVE) and amplification. The titrations which gave best recoveries for the enriched beads i.e. range between 5 to 15%

(best ~ 8%) of the original bead input were selected for the large volume emulsion (LVE) which resulted in clonally amplified library ready for sequencing.

An aliquot of the DNA library was thawed and the correct volume of DNA library (per library preparation and the equation below) was added to each tube of Capture Beads by using the following formula:

µl of library needed = molecules per bead desired X no. of beads per tube* / per tube library concentration (molecules/µl)

where, * 35 X 10^6 for LVE; 2.4 X 10^6 for SVE

The % bead enrichment was calculated by counting 3-5 µl aliquot of the beads with a particle counter using microscope using the formula:

$$\% \text{ Bead Enrichment} = \frac{\text{Number of enriched beads X 100}}{\text{Total input beads*}}$$

where, * 35 X 106 beads for LVE; 2.4 X 106 beads for SVE per emulsion

Percent enrichment values between 5 and 15% (best ~8%) are indicative of libraries yielding good sequencing results.

3.2.3.2.1 DNA Library Capture

The DNA capture beads were prepared and correct volume of the DNA library that will provide optimal amplification was added, following the equation mentioned below:

µl of library needed = molecules per bead desired X no. of beads per tube*/ per tube library concentration (molecules/µl)

where, * 35 X 10^6 for LVE; 2.4 X 10^6 for SVE

Live amplification mix was added to each tube of captured library mix.

3.2.3.2.2 Emulsification

The content of each captured library was added to the emulsion tube containing emulsion oil and shaken in TissueLyser.

3.2.3.2.3 Amplification

After emulsification, the emPCR amplification mixes were pipetted into 96-well thermocycler plates at 100µl per well and plates were tapped to dislodge air bubbles. The plates containing the emulsified amplification reactions were placed into the thermocycler and amplification was carried out.

3.2.3.2.4 Bead Recovery

The amplified emulsion from each thermocycler well was drawn and washed with isopropanol.

3.2.3.2.5 Bead Washes and Recovery for SVE

The beads were washed with isopropanol followed by wash with 1X Enhancing Fluid XT.

3.2.3.2.6 DNA library bead enrichment

Melt solution was prepared and added to each sample tube followed by the addition of Annealing Buffer XT.

3.2.3.2.7 Enrichment of the DNA Carrying Beads

Enrichment beads were prepared and added to each tube of amplified DNA beads. The beads were washed with 1 ml of 1X Enhancing Fluid XT per tube, until there were no visible beads remaining in the supernatants.

3.2.3.2.8 Collection of the Enriched DNA Beads

The enriched beads were resuspended in melt solution. DNA bead recovery was carried out and the bead pellet was resuspended in Annealing buffer XT.

3.2.3.2.9 Sequencing Primer Annealing

Sequencing primer in appropriate amount was added to the enriched beads and placed at 65^0 C on heat block followed by placing on ice. Annealing buffer XT was added to each bead pellet and % bead recovery was determined by using following equation:

The % bead enrichment was calculated by counting 3-5 µl aliquot of the beads with a particle counter using Leica Microscope using the formula:

$$\% \text{ Bead Enrichment} = \frac{\text{Number of enriched beads X 100}}{\text{Total input beads*}}$$

where, * 35 X 10^6 beads for LVE; 2.4 X 10^6 beads for SVE per emulsion

Percent enrichment values between 5 and 15% (best ~8%) are indicative of libraries yielding good sequencing results.

3.2.3.3 Sequencing

Sequencing of clonally amplified, bead-immobilized DNA library was carried out by using GS FLX Titanium Sequencing Kit XLR70 on Genome Sequencer FLX Instrument. The GS FLX Titanium Sequencing Kit XLR70 was used in combination with the matching GS FLX Titanium Pico Titre Plate Kit 70 × 75 mm.

3.2.3.3.1 Pre-Wash

The GS FLX Titanium Sequencing Instrument was started and logged in to open the windows. The Pre-wash cassette was prepared by placing the GS FLX Pre-wash Tube Holder and the Pre-wash Tubes on top of the Reagents cassette. The prewash was launched by selecting Pre-wash run in the instrument panel of the GS FLX.

3.2.3.3.2 Preparation of Bead Buffer 2 (BB2)

To the pre-chilled Titanium Bead Buffer, Titanium Supplement CB and Apyrase were added. They were mixed by gentle inversion between each addition and kept on ice.

3.2.3.3.3 Preparation of Pico Titer Plate (PTP) and Bead Deposition Device (BDD)

The PTP ID number was noted and the same was submerged in BB2 and left for at least 10 min at RT. The bead loading gasket and the BDD were washed with MilliQ water and air dried.

3.2.3.3.4 Preparation of the Packing Beads

Packing beads were prepared in BB2 solution and was resuspended in of BB2 after third wash.

3.2.3.3.5 Preparation of Sample and Control DNA beads

The number of sample DNA beads required for the gasket was determined as the volume of DNA library bead suspension to be used was based on the number of beads needed and the concentration of the library, in beads/µl (determined at the end of the emPCR Amplification procedure). The proper amounts of Control DNA beads were added.

3.2.3.3.6 Preparing the Enzyme and PPiase Beads (Bead Layers 1, 3 and 4)

The Enzyme and PPiase Beads were washed with BB2. The beads were resuspended by addition of BB2 to each tube and mixed.

3.2.3.3.7 Combining the DNA and the Packing Beads (Bead layer 2)

Appropriate volumes of washed Packing Beads and BB2 were transferred to the tubes containing the DNA beads.

3.2.3.3.8 Assembly of the BDD with the PTP Device and the Bead Loading Gasket

The soaking PTP device was removed from the tray by handling with the edges. The BDD was assembled with the PTP device and the Bead loading gasket.

3.2.3.3.9 Depositing the Four Layers of Beads on the PTP Device

3.2.3.3.9.1 Deposition of Bead Layer 1: Enzyme Beads Pre-Layer

Bead layer 1 was loaded into each of the 4 regions of the PTP device through the port holes. The PTP was spun to deposit the beads into the wells.

3.2.3.3.9.2 Deposition of Bead Layer 2: DNA and Packing Beads

The supernatant of layer 1 was removed from the PTP with a pipette through the port holes and bead layer 2 was loaded into each of the 4 regions of the PTP device. The PTP was spun to deposit the beads into the wells.

3.2.3.3.9.3 Deposition of Bead Layer 3: Enzyme Beads Post-Layer

The supernatant of layer 2 was removed from the PTP with a pipette through the port holes on the BDD top after removing the seals. The tube of bead layer 3 was loaded into each of the 4 regions of the PTP device through the port holes on the BDD top (ensuring that no air bubbles were incorporated). The PTP was spun to deposit the beads into the wells.

3.2.3.3.9.4 Deposition of Bead Layer 4: PPiase Beads

The supernatant of layer 3 was removed from the PTP with a pipette through the port holes on the BDD top after removing the seals and bead layer 4 was loaded into the regions of the PTP device through the port holes. The PTP was spun to deposit the beads into the wells.

3.2.3.4 The Sequencing Run

3.2.3.4.1 Removing the Pre-Wash Cassette and Cleaning the Fluidics Area Deck

The pre-wash reagents cassette was removed and the pre-wash tubes were discarded and the outside of the cassette were wiped. The fluidics area deck was wiped with 50% ethanol and allowed to air dry.

3.2.3.4.2 Preparing and Loading Sequencing Reagents Cassette

Titanium Supplement CB and DTT were added to each of the 4 bottles of Titanium Buffer CB provided in the kit and mixed gently by inversion. The bottles of supplemented Titanium Buffer CB and the sequencing reagents tray (cold) were placed in the reagents cassette. The entire sequencing tray was mixed by inverting atleast 20 times till no undissolved particles could be seen. The reagent cassette was loaded into the instrument and the exterior fluidics door was closed.

3.2.3.4.3 Cleaning the PTP Cartridge and the Camera Faceplate

The surface of the cartridge, camera faceplate and surface of the PTP cartridge were wiped.

3.2.3.4.4 Loading and Setting the Run Script and Other Run Parameters

The Run name and group was entered and then fourth Run Wizard window was opened and the appropriate sequencing kit i.e. XLR

70 Ti was selected for the run The Pico Titer Plate region was selected to select the number of cycles appropriate for the run whereby 200 cycles (produces reads of about 350 - 450 bp) were selected. Appropriate Run processing type was selected. Full processing was clicked to conduct both the Image Processing (i.e. “Raw wells” data files) and Signal Processing (Read flowgrams and basecalls) during the run time. The PTP device was slided into the PTP cartridge frame, face down and the camera door was closed. The sequencing run was started.

3.2.3.4.5 Data Processing during Sequencing Run

The GS Sequencer governs the sequencing run itself, whereby the raw images were acquired as digital images (PIF files). The raw data was processed by the GS Run Processor, which was composed of two parts: image processing (generation of pixels/ image in Composite Wells Format (CWF) Files) and signal processing (generation of flowgrams containing read basecalls and per-base quality scores in Standard Flowgram Format (SFF) files). The time taken for the completion of run was about 8-10 h.

3.2.3.5 Post Run Data Analysis

3.2.3.5.1 Assembly and Mapping

The Post-Run data analysis was done after the completion of the run on a dedicated data processing computer (cluster) i.e. DataRig which is a Linux-based computer dedicated to running Genome Sequencer FLX System data processing and data analysis software. The reads were obtained which were processed for removal of MID adaptor sequences.

3.2.3.6 Bioinformatics analysis

EGAssembler web based tool was applied to eliminate low quality, low complexity and repetitive sequences. Repeat masking was

done to mask the repetitive sequences. The reads were assembled into contigs and singletons (unigenes) using CAP3 programme. These unigenes were blasted in BLAST2GO for characterization of genes and KEGG metabolic pathway annotations were carried out.

Four different parameters of masking were evaluated for assembling the reads and out of four parameters; only one was used for further analysis. Four different parameters namely contigs assembled without masking the reads, contigs assembled after using Repeat masker, contigs assembled using Repbase by masking with rice repeat database and contigs assembled by masking with sorghum repeat database of TIGR.

3.2.3.7 Expression analysis

The expression was determined as how many times a unigene was represented in each of the library based on the number of reads for each unigene count. Determination of genes which were common in the two transcriptomes, versus being specific to a library was carried out based on the unigenes count (Barakat *et al*., 2009).

IV RESULT AND DISCUSSION

The results obtained in the present investigation are presented as follows:

4.1 Pathogen inoculation and Hypersensitive Response (HR)

The pathogen inoculum was prepared from infected pearl millet leaves (Plate I) and the inoculum load was adjusted to $4x10^4$ zoospores/ml using haemocytometer. The inoculum was stained with lactophenol and observed for sporangium and active zoospores under microscope (Plate II). Hypersensitive response was observed in two days after germination (DAG) grown seedlings (Plate III) inoculated with inoculum.

The response was observed as brown necrotic streaks on the coleoptiles, shoot tip and root tip region of seedlings. The result of HR, 36 hours post inoculation (hpi) is presented in Plate IV. The resistant genotypes showed early HR than the susceptible genotype. The HR in resistant inoculated seedlings was rapid with necrosis appearance as early as six hpi followed by the susceptible inoculated seedlings showed necrosis after 18 hpi. Size and number of necrotic streaks that appeared on the seedlings were 1.06% more in case of resistant inoculated than the susceptible inoculated after 36 hpi. The visual observation of HR of pearl millet seedlings are presented in Table 4.1. The HR observed was 89% and 84% in resistant inoculated and susceptible inoculated seedlings respectively whereas control seedlings showed negligible HR. This can be attributed to local defense in a form of in the seedlings (Heath, 2000).

The HR seedlings showed brown streaks which was in coherence with the earlier observation of Xingquan (2010) in the wheat-*Puccinia*

striiformis pathosystems. It can be further noted that the HR deprived downy mildew of nutrients and/or release toxic molecules, thereby confining its growth to a small region of the pearl millet seedlings, consequently providing resistance to the downy mildew pathogen.

Table 4.1 Visual observation of Hypersensitive Response of pearl millet seedlings at 36 hpi

Sample name	Resistant inoculat	Resistant control	Susceptible inoculate	Susceptible control
Total Number of seedlings showing HR (out of 100)	89	3	84	5
SEm.	0.51			
C.D $_{0.05}$	1.56			
C.V.%	8.93			

4.2 Determination of Peroxidase activity

The results of peroxidase activity expressed as change in O.D./min/g fresh tissue are presented in Table 4.2. Resistant inoculated (2.59) showed increased peroxidase activity than resistant control (1.08), susceptible inoculated (1.06) and susceptible control seedlings (0.99). A 2.4 fold increase in peroxidase activity was observed in resistant inoculated seedlings than control whereas a slight increase was noted in susceptible inoculated over its control. A correlation between disease reaction in form of HR and peroxidase activity was observed. The results affirmed with the results of Shivkumar *et al*., (2003), that peroxidase activity is associated with reduction in the rate of pathogen multiplication and spread and thus can be used as a biochemical marker of resistance against downy mildew disease.

Table 4.2 Peroxidase activity of pearl millet seedlings at 36 hpi (change in O.D./min/g fresh tissue)

Sample name	Resistant inoculated	Resistant control	Susceptible inoculated	Susceptible control
Mean	2.59	1.08	1.06	0.99
SEm.	0.11			
C.D $_{0.05}$	0.33			
C.V.%	15.11			

4.3 cDNA Library preparation

4.3.1 Total RNA isolation

Total RNA was isolated from resistant inoculated, resistant control, susceptible inoculated and susceptible control pearl millet seedlings, followed by quality and quantity check on nanodrop. The nanodrop readings are presented in Table 4.3.

Table 4.3 Qualitative and quantitative analysis of Total RNA from pearl millet seedlings

Sample name	ng/µL	260/280	260/230
Resistant inoculated	3013.7	1.97	1.53
Resistant control	2776.3	2.01	1.73
Susceptible inoculated	3433.7	1.93	1.52
Susceptible control	4210.6	1.91	1.63

The quality of total RNA extracted (260/280 ratio) ranged from 1.91 to 2.01, which is in agreement with the normal purity standards of RNA (Chomczynski and Mackey, 1995).

4.3.2 mRNA isolation

From total RNA, mRNA was isolated by oligo d(T) tracking through magnetic bead which was fragmented and assessed through Pico chip before and after fragmentation (Fig 4.1 and Fig 4.2 (a-d)). The nanodrop readings of mRNA concentrations are presented in Table 4.4. The concentrations of mRNA before and after fragmentation are presented in Table 4.5 and Table 4.6 respectively.

Table 4.4 Qualitative and quantitative analysis of mRNA from pearl millet seedlings

Sample name	ng/µL	260/280	260/230
Resistant inoculated	95.78	1.97	1.81
Resistant control	98.68	2.04	1.89
Susceptible inoculated	112.25	2.04	1.86
Susceptible control	162.24	2.02	1.90

The quality of mRNA extracted (260/280 ratio) ranged from 1.97 to 2.04 which is in agreement with the normal purity standards of RNA (Chomczynski and Mackey, 1995).

Table 4.5 Qualitative and quantitative analysis of mRNA before fragmentation from pearl millet seedlings on RNA pico chip

Sample Name	mRNA before fragmentation		
	RNA Area	Concentration pg/µl	rRNA ratio 28S/18S
Resistant inoculated	453.2	3103	0.7
Resistant control	528.9	3622	0.6
Susceptible inoculated	463.9	3176	0.3

Susceptible control	428.8	2936	0.1

Fig 4.1,

Fig 4.2

Table 4.6 Qualitative and quantitative analysis of mRNA after fragmentation

from pearl millet seedlings on RNA pico chip

Sample Name	mRNA after fragmentation		
	RNA Area	Concentration pg/µl	rRNA contamination
Resistant inoculated	281.7	3019	0.0 %
Resistant control	559.1	5992	1.3%
Susceptible inoculated	380.6	4079	0.0%
Susceptible control	665.8	7136	3.4 %

The resistant inoculated and susceptible inoculated showed zero percent contamination of rRNA whereas resistant control (1.3%) and susceptible control (3.4%) showed very less rRNA contamination respectively. The resulted fragmentation was appropriate, that differed in shape with a narrower size distribution with a majority of mRNA between 450 and 1200 nucleotides which was in accordance with the manufacturer's protocol. The fragmented mRNA was used for first stand cDNA synthesis. Double stranded cDNA was synthesized from single stranded cDNA, followed by quality and quantity check on nanodrop. The nanodrop readings are presented in Table 4.7.

Table 4.7 Qualitative and quantitative analysis of double stranded cDNA (1:1 dilution)

Sample name	ng/µL	260/280	260/230
Resistant inoculated	25.4	1.88	1.84
Resistant control	23.4	1.71	1.68
Susceptible inoculated	48.8	1.77	1.80
Susceptible control	23.8	1.89	2.02

The quality of cDNA extracted (260/280 ratio) ranged from 1.71 to 1.89 which is in agreement with the manufacturer's protocol.

Four libraries were prepared after Multiplex identifiers (MID) ligation for resistant inoculated, resistant control, susceptible inoculated and susceptible control respectively. All the four libraries were quantified fluorometrically and the results are presented in Table 4.8. The same DNA libraries were quality checked and quantified through DNA High sensitivity chip on Bioanalyzer. The cDNA molecules in four libraries were calculated and diluted to 1 X 10^7 molecules/µl in TE.

Table 4.8 Quantification of pearl millet cDNA libraries by Fluorometer

Sample Name	Fluorescence	Molecules /µl
Resistant inoculated library	7.481	1.75E8
Resistant control library	6.064	1.47E8
Susceptible inoculated library	5.345(below the lower limit of standard)	Considered as 1E8
Susceptible control library	118.5	23.9E8

4.4 Emulsion based clonal amplification and sequencing run

The enriched beads through emPCR were loaded onto the Pico Titre Plate 70 X 75 mm and sequencing was carried out in 50% of the Pico Titre Plate. The overall sequencing data generated was 34 Mb for all the four multiplexed samples with a total of 134788 raw reads (34890767 bases). The maximum read length was 604 bases with an average read length of 258 bases. The results are presented in Table 4.9.

Table 4.9 Sequencing Data of pearl millet cDNA libraries

Sample	Resistant inoculated library	Resistant control library	Susceptible inoculated library	Susceptible control library	Total
Number of reads	35512	50262	23971	25043	134788
Total number of bases	8829575	13331593	6514365	6215234	34890767
Average length of reads (bases)	247	265	272	248	258
Size of reads (bases)	27-562	23-604	20-596	24-564	20-604
Number of low complex reads	2916	3990	2365	1908	11179
Number of high quality reads	32596	46272	21606	23135	123609

4.5 Bioinformatics analysis

4.5.1 Processing of sequencing data

The data was processed into fasta files and obtained in the form of sequencing reads. MID adapter sequence removal were performed by in-house Perl scripts to obtain clean reads. EGAssembler web based tool was applied to eliminate low quality, low complexity and repetitive sequences keeping default parameters. Out of 134788 reads, 11179 reads (8.29%) were eliminated, 198 reads (0.14%) were trimmed and 123609 (91.70%) reads were used for further processing. The read data was compared via four different assembly parameter and the results are presented in Table 4.10 and Fig 4.3.

By comparing the unigenes assembled from all the four parameters based on average length of contigs, number of singletons generated, blast hits, mapping and annotation, the contigs assembled without repeat masking gave the best results in terms of blast hits (Fig 4.4) and the number of unigenes annotated (Fig 4.5) were more as

compared to other assemblies. Hence, the reads without repeat masking were employed further for generating contigs and singletons.

Table 4.10 Comparison of four different assembly parameters of Resistant

inoculated library sequences

Parameters	**Without Masking**	**Masked with Repeat Masker**	**Masked with rice Repbase data**	**Masked with Sorghum TIGR repeat database**
Initial valid sequence	32596 (63 trimmed)	32596 (63 trimmed)	32596 (63 trimmed)	32596 (63 trimmed)
Number of reads masked	Not applicable	23282 (65.56%) (as small RNA)	6630 (as Repeat element and plastid sequence)	10882 (as DNA transposons)
Reads used for assembly	32596	9314	28882	21714
Number of contigs	527	473	529	588
Average length of contigs (bases)	425	341	422	380
Range of contigs (bases)	104-5423	100-2217	104-5109	100-2123
Number of singletons	1612	1368	1588	2330
Number of Unigenes	2139	1841	2117	2918
No Blast Hits	822	796	813	950
No Mapping	458	262	451	1308
No annotation	57	46	56	50

These cleaned reads were subjected to CAP3 online programme for clustering and assembly. Contigs and singletons were generated at a stringency level of 80% homology and 40 bp overlap. The reads thus assembled into contigs and singletons were called the unigenes. The results are presented in Table 4.11.

Fig 4.3, 4.4

Table 4.11 *De novo* assembly of all pearl millet cDNA libraries sequence by

CAP3 with 40 bp overlap and 80% identity

Parameters	Resistant inoculated library	Resistant control library	Susceptible inoculated library	Susceptible control library	Total
Number of reads	35512	50262	23971	25043	134788
Total number of bases	8829575	13331593	6514365	6215234	34890767
Trashed sequence	2916	3990	2365	1908	11179
Number of reads used for assembly	32596 (63 trimmed)	46272 (59 trimmed)	21606 (63 trimmed)	23135 (13 trimmed)	123609 (198 trimmed)
Number of contigs	527	410	348	168	1453
Average length of contigs (bases)	425	438	489	454	451.5
Range of contigs (bases)	104-5423	100-3930	106-4536	145-4348	100-4536
Number of singletons	1612	1636	1077	526	4851
Number of Unigenes	2139	2046	1425	694	6304

The reads utilized for generating unigenes for all the four libraries were 32596 (26.37%), 46272 (37.43%), 21606 (17.47%),

23135 (18.71%) and the number of unigenes generated were 2139, 2046, 1425 and 694 respectively.

4.5.2 Blast2GO

The unigenes were loaded onto the Blast2Go interface for functional annotation with a search threshold of 1e-6. The results are presented in Table 4.12.

Table 4.12 Functional annotation of pearl millet cDNA unigenes

Parameters	Resistant inoculated library	Resistant control library	Susceptible inoculated library	Susceptible control library	Total
Number of Unigenes	2139	2046	1425	694	6304
Number of BLAST hits	1317	1356	825	400	3898
No Blast Hits	822	690	600	294	2406
No Mapping	454	481	296	143	1374
No annotation	54	58	38	18	168
Annotation	809	817	491	239	2356

Most of the Evidence code in the present study belonged to the Inferred from Electronic Annotation (IEA) which is presented in Fig 4.6.

Pearl millet sequences gave maximum blast hits with *Oryza sativa* followed by *Zea mays, Sorghum bicolor, Vitis vinifera* and *Hordeum vulgare* (Fig 4.7). Rice has a high degree of synteny with genomes of other cereals plants, such as maize, wheat, barley, pearl millet and other grasses because their genomes share a considerable similarity in their organization, as well as sequence similarity (Gale and Devos, 1998).

BLAST search analysis across all the four libraries revealed highest homology with the sequences from *Sorghum bicolor*, *Zea mays*, *Oryza sativa*, *Hordeum vulgare* (Fig 4.8). This is supported through comparative genetic mapping of cereal crops that both gene contents and/or gene orders are largely conserved over the evolutionary history of the grasses (Moore *et al*. 1995, Gale and Devos, 1998). Thus, for conserved genomic regions, map location of a sequence in one species can be used to predict the location of the sequence across much of the grass family (Yadav *et al*., 2008).

Fig 4.5, 4.6

Fig 4.7, 4.8

4.6 Functional annotation of resistant and susceptible genotypes

In the process of functional annotation of the putative encoded proteins, unigenes were classified into biological, molecular and cellular function based on the GO terms.

4.6.1 Biological processes

Using the GO ID information, the sequences were classified into subsets based on biological processes and is represented in Fig 4.9 for each library. The functions of genes identified covered various biological processes such as metabolic, catabolic processes, membrane transport, transcription, translation, protein folding, and others.

A major subset of sequences belonged to the generation of precursor and energy process followed by protein modification, cellular

amino acid derivative and response to stress. A larger number of genes involved in response to biotic and abiotic stimuli and stresses were identified in resistant inoculated library as compared with other libraries. Response to biotic and abiotic stimulus was highest in resistant inoculated library. This can be due to the crosstalk between the biotic and abiotic defense mechanism (Koornneef *et al*., 2008), during the defense mechanism in response to the downy mildew pathogen, various other abiotic stress defenses could crosstalk leading to their expression too. The dehydration responsive element binding (DREB) transcription factor primarily functioning in abiotic stress was found to be expressed in the resistant inoculated library. The secondary metabolic processes were highest in resistant inoculated library than other three libraries suggesting the synthesis of secondary metabolites through activation of secondary metabolite pathways like shikimate acid pathway, pentose phosphate pathways in response to downy mildew pathogen which is affirmed by the studies of Narina *et al*., (2011) and Carlos *et al*., (2008).

Fig 4.9

4.6.2 Molecular processes

In the molecular function subset, nucleotide binding, protein binding and transporter activity were among the most represented molecular function categories. GO-annotation function distribution is presented in Fig 4.10.

Fraction of genes involved in protein kinase activity, electron carrier activity and transcription factor activity were highest in resistant inoculated library. Several sequences were annotated as protein kinases which can be approved to have a definite role in disease signaling. The presence of genes for synthesis of cell wall precursors and secondary metabolites was seen to be highly induced due to biotic stress. An increase in molecular functions in nucleic acid protein binding and transcription factors, in resistant inoculated library can be qualified for its involvement in defense signaling due to downy mildew infection. Genes encoding helicases, bromodomain, snf related proteins, histone deacetylases, splicing factors were highly expressed in resistant inoculated library suggesting the onset of various gene regulating pathways and cascade of reactions after sensing the downy mildew infection for the transcription of defense related proteins. The results obtained in the present study are in agreement with the results of Balaji *et al*., (2007)**,** where the highest percentage of up-regulated genes were those involved in transcription, signaling, stress and transport in resistance response triggered by recognition of the *Xanthomonas* type III effector *AvrXv3* in tomato.

4.6.3 Cellular component

In the cellular component subset, major cellular components involved in the biological and molecular functions were plastid, mitochondria, protein complex, cytoplasmic bound vesicles and ribosomes (Fig 4.11). The most abundant sequences in the libraries corresponded to the genes encoding photosynthesis-related proteins such as RuBisCO, fructose-1, 6 bisphosphate aldolase and chlorophyll a/b-binding protein. The oxidative defense pathways were activated in response to the pathogen which was revealed by the presence of enzymes and proteins like cytochrome c oxidase, cytochrome P450, oxygen evolving proteins, ascorbate peroxidase, esterase, catalase, superoxide dismutase etc. (Swarbrick *et al*., 2008). In the cytosol and mitochondria, NADP-isocitrate dehydrogenases; enzymes that generate NADPH; key redox compounds in the soluble phase of the cell are NAD, NADP all of which interact strongly with reactive oxygen. The processes taking place in ribosome fraction of cell was highest for resistant inoculated library suggesting hasty translation of proteins in response to the biotic stress.

4.7 Candidate genes involved in pearl millet response to *S. graminicola* infection

The genes involved in encoding enzymes in defense pathways, defense related resistance proteins, signaling molecules and transcription factors were identified in the present study which are presented in Table 4.13.

Genes encoding the pathogenesis related proteins such as endochitinase, glucanase, thaumatin, ribonuclease, thionine, peroxidase, were found in the present study.

The resistance related proteins included rav like proteins, expansin, tubby proteins, ferritin, fiddle like proteins, atp adp transporter proteins and ankyrin repeat proteins.

Genes for enzymes involved in defense pathways incorporated genes for acid phosphatase, phenyl ammonia lyase (PAL), lipoxygenase, hydrolases, h^+ ATPase, allene oxide synthase, chalcone synthase, glucosidase, esterase lipase domain, gsd1-motif lipase, glutathione S-transferase, dihydrofolate reductase, cysteine proteases and oxidative pathways involved superoxide dismutase (SOD), oxygen evolving proteins, catalase, ascorbate peroxidase and esterase.

Fig 4.10

Fig 4.11

Table 4.13

Genes encoding various kinases included adenosine kinase, amino transferases, Ionositol polyphosphate 2 kinase, protein kinases, shaggy protein kinases. Early defense response genes, immediate early fungal elicitor proteins, *mlo* protein, stress enhanced protein, *rxo1* disease resistance protein were also identified in the present study.

Various transcription factors involved in biotic interaction between pearl millet–downy mildew included DEAD box, WRKY25, homeodomain leucine zipper, gata zinc finger family, ring finger family, heat shock transcription factors, DREB, F-box and creb.

The results affirm with the earlier observations of Manickavelu *et al*., (2010); Swarbrick *et al*., (2008); Barakat *et al*., (2009) and Jiang *et al*., (2007) where the products of resistance genes were defense

related enzymes, pathogenesis related proteins, receptor-like kinases, and resistance proteins typically containing NBS LRR domains, involved in pathogen recognition and signal transduction. It is possible that such receptor-like R gene homologues could be functionally important in the resistance of pearl millet against downy mildew.

- **Contig and read sequence of annotated genes**

Contig53 GO:0009536 rav-like protein

GTAGTAGCATACTAGTCAAAGTCAACAGCAGCTGAGCAGCCGGAGGTCGAGAGCGCGCGC
GGGAGCCCCCATGGAGTTCGCGCACGCACACGGCGGGGGTCGAGGATTCCCGAGAGGGGC
CCCGCGGCGCCGGAGGCGGCGCCAGCGCGGCGTGGGTGGAGAAGGAGCTACATGTTCGAG
AAGGTGGTGACCCCCAAGCGACGTGGGGAAGCTCAACCGCCTGGTCATCCCCAAGCAGCA
CGCGGAGCGCTACTTCCCCGCGCTGGACCCGTCGGCGGCGGCCGCGGCGGGCGCTGGCGG
CAAAGGGCTGGTGCTCAGCTTCGAGGACCGGTCGGGGAAGGCGTGGCGGTTCCGGTACTC
GTACTGGAACAGCAGCCAGAGCTACGTGATGACCAAGGGGTGGAGCCGCTTCGTCAA

Contig150 GO:0042549 oxygen-evolving enhancer protein 1

CTCAAAGCTTTTCATACACTTCCCCATCGATCATGGACGGTCTAGTTCGACTCGAGCTGC
GCGTACCAGACCCCCTGGATTTTCACATCCTTGGGTGCCTTGGCCCCAAGGTCGGTGTCC
GACGGCTGCACACTCTCGAAGACACCGATGACCTCGCCAGTTTCCGGGTTGCTCTTGGTG
ACGCTCAATGTGATATTGCCCGTGGACGACGCAGCGTTCTTGATGTTCTCCTTCACGAGC
TCCTCCTCGTCTCCCCTGCCTCCGGCGGGGAGCGCGACGGCGTTGTCGTACCCGGTAGAG
CCACCACGGCCCTTGGGGTCCAGGAAGGAGGAGCCACGGTAGCTGGGCACGAGGAAGGGA
CCGCCGAAGCTCTCGGGCTTGCCGGTGGCGACGAGCTGCTTGACAGTGAAGAGGAACGGC
ACGCGCTCGCCTCCCGGAAGCTGCACGGTGACGGCAGCGTAGTCGATGC

Contig228 GO:0043565 homeodomain leucine zipper protein

CCAGCGCGCGCGGCGGTCTGGAACCAGACGGCCGACCTGCGGGGCGCCGATCCCCAGCCG
TCGCGCGAGCTCGGCTTGCGCTCCGGCTCCAGCTTGTTCTCCTCCTCGAAGCTCCGCTCC
AGCAGCTGCACCTGCTCCGCTGTCAGCCGGCGCTCTTCTCCGGCGCCGGCTCGTCGTAGT
ACTCCTCCTCCATGAGCTCCTCGTGCGTCGTGAAGAAGGGCCGCTTGCCCACGCGCACCG
CGTCGTCCATGCCCAGAACCGCCATCGGGACACCTCGGAAGAAGCCGTTGCTGTTGGCGC
TGCCCCCGCCGCCGAAGAGCAGCATCTGCGCTCCGCCGCCACCGCCGCCCCGCCGCGCGC
CGCCGGAGTCGACGCCGACGCGGCCCGGATCCATGCTGATTCCCCTCCCTCCTTCCTTCT
ACGAAACTTCTCCTAGCCCCCCTTATCTCCTCCCAAACGAACGAAACGAAGCCCACCACG
AGCGCGGGCGCGCGCGCAAACACACAGTCACAGGCACACGCACGCGCGCACACACACTCC
AACGGCTGCTAGGAATCGCGCGGCCGCCGAGGCGATCGAGGGCGGGGCGTCAGATGAAGC
AGCAGGAGGAGGCGAGCTTATGGCGGAAGAAGCTGGTGCTCCAGAGCATGCGCGTGGACG
TCTTCATGGGGGTACAGCGAGAACCCCATGGCCCCCTCCGCAGGTAAACGACGCGGGTGG
ATTCCTCCGGCGAGCGGTGGCGGACTCCTCCGGGTGG

GWE1CFD01BY68H GO:0005488 dead-box atp-dependent rna helicase 3

ATTCCTCGGGTGTTGGTTATGGCGCCTACTAGGGAGTTAGCCAAACAAGTTGAGAAGGAA
ATAAAGAATCGGCACCCAAACTCAGTACAGTGTGCGTTTATGGTGGCGTTTCGTATAATG
TCCAGCAGAACGCACTCTCTCGTGGTGTTGATGTTGTTGTTGGAACACCGGGTCGCATAA

TCGATTTGATAAATGGTGTTCCAACAACAACATCAACACCACGAGAGAGTGCGTTCTGCT
GTTGTTGTTGGAACACCGGGTCGCATAATCGATTTGATAAATGGTGGAAGCCTTCAGTTG
GGGGAAGTTCAATATTTGTCCTTGATGAGGCTGACCAAATGCTTGCAGTGG

GWE1CFD01AYQLN GO:0016023 thionin-like peptide

GGCAAAAGCATGCTGCCAAGCTTGTCTGTTTGTTGCCAGCGATTCGACTTGCAAGAACAC
TTGTTGTTATCCATGTACTATGGCTGATTCTGATGTTGCTAAAATGGATGAAATGGAAGT
TCTTGTAATTGCTCAAGAAGGACAAGCCTAAAGCAACATGCTTACATTGCCACCAGCATG
ATTGCCATTTTATAATCATATGTTGTTTTGCTTCTTATTATGGCCAAGTTATGTTAATGT
TGATATCCAATAAACTGTTGACCATATGGGACAACCTCTAGGTGATCCTTATGTTGGTGT
TAACAATATGTATGTCATACATGTGTGCGTGGGCGCGCGCTCACAAGCATAGT

GWE1CFD01B3WBH GO:0005515 stress enhanced protein 1

CGCGAGACCGAGGGCCCTTAGCGTGAGGTGCGAGCAGGGAGCCAAGGGCGGCGGGCTGGA
CGTGTGGCTGAGCCGCGGCGCGATGCTGGGCTTCGTGGGGTGGTGGCCGTCGAGCTGACC
ACCGGCAAAGGGGTGCTTCAGAACGTTGGCCTGACCGCGCCGCTGCCGACGGTGGCGCTG
GGGCTCACCGGCGTGGTCGGGATCTTCACGGCCTTCATCATCTTCCAATCCGGGTCGCAG
GACTGATGGGTGCGTGGATACCTGAGGCTGATTCCCGAACAGGATTGCATCCTGAGCTGC
CGGCGCCTCCATTATTCGAGCTCTTCCTCCTCTAGACTCTAGTGCCTTCTGCCTTGTATG
TGTTTGTG

GWE1CFD01AXZU3 GO:0005515 f-box family protein

ACAGCCTTCAGTCCCCGATCCGAGATTTCTGTGCAACCCTCAAGACTAAGGCACTGCAAA
GAACCCGGGGACTTTTGTGTCAGGCGGAGAAGATTAGCATCTTTAATCTTCTTACCCAAC
TGTGAGTCAATATGGATGCACTTCCATAAAAATGGTTCATTCCGAACAGCTGCACGCAAT
GACTTGCACACCATCTCAAGAGAGAGGATTTCACGTAAGCCAAGATAGCCCAGAACAAAA
CCCATTCCCTCATGTGCATCATTTCCTTCTTGGACAGGAGCAGCATCAACTGTATCACAG
CATACC

GZL4SJN01D3VOT GO:0016757 avr9 cf-9 rapidly elicited protein 231

GGCCGCCATGGAGCCCCGGAGGTAGTGCGCGTCGAGCGTCATGGCGATGTGGACGAGGCC
CGGGTCGCAGACCCCCGCGCCCGCCACGGGCGCCGGGCACCCCTCCCCGTTCCGGTACTC
GGGCGCCTCGGCGAACCTGGGCAGCCCCGCGGCCGCCGATTGGGCCGATAACAACATCGC
CGCCAGCACCAGGCCGACCCACATCGCCGGGGTGGCCGCGCCCCAT

Contig363 GO:0016023 mlo-like protein 4

GCAGCTACAGCACACTGCCTCTTAATGTGATCATTTCTCAGATGGGATCCAAGTTCAAGA
AGTCACTGGTGTCGGAGAACGTGAGGGAGTCGCTCCACAGCTGGTGCAAGAGGGTCAAGG
ACAGGAACAGGCAGAACCCGCTC

GWE1CFD01BVOHK GO:0003700 myb family transcription factor-related protein

CGGTGGACGGCCGACCTGCACGAGCGCTTCGTCGACGCCGTCGCCCAGCTCGGCGGGCCT
GAGAAAGCGACACCAAAAACTATCTTGAGGACAATGGGTGTCAAGGGGCTTACGCTTTTC
CACTTGAAGAGCCACCTTCAGAAATACAGACTAGGCAAACAATCTGGTAAAGAGGGGTCA
GAGCAGTCTAAAGATGCATCCTATCTTCTAGATGCTCAAAGTGGAATGAGTGTATCCCCT
AGAGTTGCTGCCCAGGATGTGAAAGAAGTCAAGAAGTCAAAGAAGCACTGAGAGCACAGA
TGGAAGTGCAACGGAGACTGCATGAACAAGTGGAGGTCCAGAAGCGTGTGCAGATCAGAA
TGGAAGCGCTTCAGAGTACATTGATAGTATTCTGGAGAGTGCATGCAAGATGGTCAC

GWE1CFD01B5SBE GO:0006468 enhanced disease resistance 1

CTTCCTTCATCTCCCTTAACGCATTATCAAACTTATGGTCAATCACTATCACTTCAAACC
CAAGATCCCCAATGCTCATCTGAAGCTCAGAAAGCGATGGGATCTTTTTCTGCCTAGAAG
ACTCCACAGAGAGTCCACATATGTCATAGAATCCATCAATACTTTCNCATTGTAATCAAG
AAAGTTGTATTCCTGGTATCGCCGTGAGAGCGACTCCGCGGTTTCTCTCC

GWE1CFD01BQ3TZ GO:0000166 rxo1 disease resistance protein

AGATATCATCCAATATGATCAAGTACCGCCTTTTATCCAGAATTTTGATCAATTCTACGC
TTAGTTCTTCTGTACCATTCATTGTTATAGCATCAAATTCTTTCTTGTTTTCTGCAAGTA
GATCTCTCAACAGGGTCTTCCAAATATCTTCCAATTTATATGACTGAGATACAAGAAACC
CATGCACGGCAAT

4.8 Transcriptome comparison between infected and control tissues within resistant and susceptible genotypes

To determine the effect of infection by the downy mildew fungus on gene expression in resistant and susceptible genotypes, comparison between the control and infected tissues within resistant and susceptible genotypes was carried out. The unigenes differentially expressed in resistant inoculated and resistant control were 247 (15%) and 234 (14%) respectively; the genes expressed in common were 1145 (71%). The unigenes differentially expressed in susceptible inoculated and susceptible control were 226 (31%) and 71 (10%) respectively; the genes expressed in common were 433 (59%). Comparison between the resistant and susceptible genotypes depicted more number commonly and differentially expressed genes in resistant genotype than the susceptible genotype. The reason behind this might be the expression of more genes in resistant than the susceptible genotypes (Fig 4.12 (a-b)).

Some of the genes were specifically and differentially expressed in the resistant inoculated and resistant control library. The differentially expressed genes are shown in Table 4.14.

Avr9cf- related elicitated protein, snf proteins and stress enhanced proteins were found to be exclusively expressed in “resistant inoculated” library in response to downy mildew infection. This is in congruence with the study of Kamoun (1999) where an *Avr9* peptide of the fungal pathogen *Cladosporium fulvum* elicited the HR in tomato and tobacco. In pearl millet also, the downy mildew pathogen must have elicited such a protein as a part of HR.

The genes specifically expressed in the “resistant control” library included enhanced disease resistance (*edr*) genes, *mlo* proteins, *rxo1* disease resistant protein, armadillo proteins, dreg2 protein, knotted like proteins, PHD finger type proteins, tbc domain containing proteins.

Enhanced disease resistance 1 (*edr1*) was previously reported by Tang *et al*., (2005) to function as a negative regulator of disease resistance in *Arabidopsis*, in pearl millet–downy mildew interaction, the *edr* gene expression in the resistant library could be attributed as a part of defense mechanism. In pearl millet downy mildew interaction, the *rxo1* gene could trigger pathogen specific HR in resistant inoculated library as observed by Zhou *et al*., (2010) in rice (*X. oryzae)* interaction where the *rxo1* gene was responsible for HR. *mlo* was revealed to arrest fungal development by cell wall appositions in resistance interaction between barley and *Blumeria graminis* by Huckelhoven *et al.,* (2001) which is affirmed in the present interaction. Thus, the presence of these genes in resistant library can be ascribed to the attempt to cease the growth of *S. graminicola* as a part of HR.

The genes present in both the resistant inoculated and resistant control whose level of expression differs suggests that, in incompatible plant-pathogen interaction the induction of resistance by the genotype is in higher quantum against the pathogen.

In the susceptible inoculated library, genes for WD 40 proteins, armadillo proteins, expansin proteins, gras family protein, hedgehog-

interacting protein, tubby protein, F-box proteins, heat shock proteins, LRR, cell wall hydrolase, caffeoyl 3-O-Methyl transferase, peroxidase, PAL, cellulose synthase, chitinases, cyt P450, cysteine protease, glucanase, allene oxide synthase, h^+ ATPase and β-glucosidase were expressed.

In the susceptible inoculated library, various transcription regulators were expressed which can be adjourned as a part of induction of defense response. The gras family of transcription regulators were also shown to be expressed in *Pseudomonas syringae*-tomato interaction studied by Mayrose *et al*., (2006). The expression of WRKY25 protein is in agreement with results of Ryu, (2006) Du, (2004) where altered expressions of WRKY transcription factors were seen upon attack of the fungal pathogen *Magnaporthe grisea*- rice interaction. This expression exclusively in the susceptible library was in synchronization with the findings of Eckey *et al*., (2004) in barley-powdery mildew interaction where the knockout mutants of WRKY increased resistance to the fungus. It can be postulated that the WRKY transcription factors are specifically induced in response to virulence in susceptible interaction.

The resistant interaction included high expression of PR genes which is affirmed by the earlier studies by Geddes (2005). Peroxidases were highly expressed in resistant inoculated than control library. This is in correlation with the results of Vergne *et al*., (2007) in a *M. grisea* resistant rice cultivar which had higher ratios peroxidase and chitinases in a microarray experiment. Chitinases are expressed in all the four tissues with a varied expression. It is highly expressed in the resistant inoculated than resistant control. This is in congruence with the observation of Huckelhoven, (2001). Lipoxygenase 2, 3, 6 were specifically expressed only in the resistant inoculated library. This is supported by earlier studies in pearl millet against downy mildew

infection by Kim *et al*., (2004); Zhu *et al*., (2006). The expression of ribonuclease in resistant inoculated library was in agreement with the results of Shivakumar *et al*., (2000). Late induction of chalcone synthase in defense response was observed which is in agreement with the observations of Christensen *et al*., (1998).

4.9 Transcriptome comparison between infected and control tissues of resistant and susceptible pearl millet genotypes

To gain insight into the differences in the response of the susceptible and resistant genotypes in response to downy mildew causing pathogen, the transcriptomes of resistant inoculated vs. susceptible inoculated and resistant control vs. susceptible control genotypes were compared (Fig 4.12 (c-d)). The unigenes differentially expressed in resistant inoculated and susceptible inoculated were 329 (25%) and 131 (10%) respectively; the genes expressed in common were 840 (65%). Comparison between the resistant and susceptible inoculated genotypes depicted more number of differentially expressed genes in resistant genotype than the susceptible genotype. This can be owed to the fact that apart from the normally expressed genes, certain genes might have induced upon downy mildew pathogen infection in pearl millet. The unigenes differentially expressed in resistant and susceptible controls were 438 (41%) and 58 (6%) respectively; the genes expressed in common were 560 (53%). More number of differentially expressed unigenes was found in resistant control than susceptible control, the expression of unigenes in common deciphers the basic metabolic and cellular activities in the pearl millet genotypes.

The list of genes differentially expressed is presented in Table 4.14.

Fig 4.12(a-d)

Table 4.14 Differential and specific expression of genes in pearl millet genotypes

Unigenes	Resistan	Resista	Susceptibl	Susceptib

	t inoculated	**nt control**	**e inoculated**	**le control**
Acid Phosphatase	+	-	-	-
Adenosine Kinase	+	-	-	-
Allene oxide	+++	-	+	-
Amino transferase	+	-	-	-
Ankyrin repeat proteins	+	-	-	-
Arginosuccinate synthase	+	-	+	-
Aspartate kinase	+	-	+	-
Aspartate kinase homoserine dehydrogenase	+	-	+	-
Atp adp translocator	+	-	-	-
h+ ATPase	+	+	+	
Avr9 cf related elicited protein	+	-	-	-
β Glucosidase	+	+	+	+
Catalase	+	+	+	+
Cbl interacting protein	+	+	-	-
Esterase lipase domain containing protein	+++	-	+	-
Esterase	+	+	+	+
Ethylene	+	+	+	-
F box family proteins	+	+	+	-
Ferritin	+	-	-	-
Fyne finger containing proteins	+	-	+	-
Gata transcription factor	+	-	-	-
Lipase hydrolase	+	-	-	-
Glutathione S transferase	+	+	-	-
Glycine rich protein	+	+	+	-
Chitinase	+	+	+	-
Gsd 1 motif lipase	+	-	-	-
Heat shock protein 70 kD	+	+	+	+
Heat shock protein 90 kD	+	+	-	-
NBS LRR	+++	+	+	+
Ubiquitin	+++	+	-	-
Ionositol polyphosphate 2 Kinase	+	-	-	-
Cell wall hydrolase	+	+	+	+
Cellulose synthase	+	+++	-	-
Chalcone synthase	+	+++	-	-
Chy and ctchy ring type proteins	+	-	-	-
C-repeat binding	+	-	+	-
NADPH oxidase	+	+	-	-
Unigenes	**Resistant inoculated**	**Resistant control**	**Susceptible inoculated**	**Susceptible control**

Cyt 450	+	+	+	+
DEAD box	+	-	-	-
DREB	+	-	-	-
dna j heat shock n-terminal domain	+++	+	-	-
Dre binding factor	+	-	-	-
Creb binding	+	-	-	-
Glucanase	+++	-	+	-
Proline rich proteins	+	-	+	-
Kinases	+	-	-	-
Lipoxygenase	+	+	-	-
MAPK	+	-	-	-
Multiple copper	+	-	-	-
Ribonuclease	+	-	-	-
Peroxidase	+++	+	+	+
Phd finger type protein	-	+	-	-
Thionine	+	-	-	-
Pentatricopeptide repeat	+	+	+	-
PAL	+++	+	+	-
Phosphoinositidyl ionositol	+	-	-	-
Phosphoglycerate kinase	+	-	+	-
Polyubiquitin 7 ubiquitin monomer	+++	+	-	-
Pyruvate kinase	+	-	+	-
rav like protein	+	-	-	-
Receptor serine threonine	+	-	-	-
Ring finger domain	+ + +	+	+	-
SAM	+	-	-	-
Serine hydroxymethyl transferase	+	+	-	-
Serine threonine kinases	+++	+	+	-
Snf-1 related	+	-	-	-
Stress enhanced proteins	+	-	-	-
SOD	+	-	-	-
Threonine	+	-	-	-
Ubiquitine ligase	+	+	+	-
Xyloglucan endotransglycosylase	+++	+	+	-
Zinc finger	+	-	-	-
BADH	-	+	-	-
Calnexin	+	-	+	-
Cell wall hydrolases	+	+++	+	-
Dreg 2	-	+	-	-
Enhanced disease resistance	-	+	-	-
Unigenes	**Resistant inoculate**	**Resistant control**	**Susceptible inoculated**	**Susceptible control**

	d			
Isochorismate synthase	+	+	-	-
Knotted	-	+	-	-
mlo protein	-	+	-	-
WRKY 25	-	-	+	-
Rxo disease resistance protein	+	+	-	-
Shaggy protein kinase	+++	+	-	-
tbc domain containing protein	-	+	-	-
WD 40	+	+	+++	+
Armadillo protein	-	+	+	-
Expansin	-	-	+++	+
Caffeoyl 3 O-Methyltransferases	-	-	+	-
Cazy	+	-	+	-
Cysteine protease	+++	-	+	-
gras family proteins	-	-	+	-
Hedgehog interacting protein	-	-	+	-
Immediate early fungal elicitor	-	-	+	-
Tubby protein	-	-	+	-
MAP kinase	+	+++	+	-
Chaperon	+++	+	-	-

+++ Highly expressed gene

\+ Expressed gene

\- Null Expression

Genes present in relatively high expression in resistant inoculated than susceptible inoculated included h^+ ATPase, β-glucosidase, catalase, cellulose synthase, chitinases, peroxidase, hsp70, LRR, PAL, ring finger, glucanase. The expression of Myb transcription factor, proline rich proteins were uniform suggesting its role in defense response.

The pathogenesis related proteins were highly expressed in the resistant inoculated than susceptible inoculated which is in agreement with the earlier results of Chandrashekhara et al., (2010); Shivakumar (2000); Kini *et al*., (2000); Shivkumar *et al*., (2008). DEAD box proteins were specifically expressed in resistant inoculated library, which

correlates with the finding of Li *et al*., (2008) in rice where DEAD box RNA helicases were shown to function in defense responses against the pathogen and oxidative stresses. Cysteine proteases were highly expressed in resistant inoculated library, suggesting that the alteration of the amino acid synthesis and nitrogen metabolism was a result of *S. graminicola* infection. Ubiquitins were expressed specifically in the resistant genotypes, highly expressed in inoculated than in the control. Glutathione S- transferase was expressed only in the resistant genotypes, highly expressed in the resistant inoculated library which corroborates with the results of Barakat *et al.,* (2009).

WD 40 proteins were highly expressed in the susceptible inoculated library than resistant inoculated which is supported by the observation of Yamamura and Shim (2008) in which the rot fungus of maize *Fusarium verticillioide* possessing *FSR1* gene containing WD 40 repeat domain at the C terminus which had a pivotal role in pathogenesis. The expression of WD 40 proteins in susceptible library could be accredited to the fungal invasion due to virulence of the downy mildew pathogen, it can be further concluded that the WD 40 proteins could be present in the downy mildew pathogen making it responsible for virulence like that of other pathogen. Its low expression in resistant library denotes the presence of certain resistance proteins which could have counterattacked WD 40 proteins.

Expansins were exclusively expressed in the susceptible inoculated library. This can be correlated to the fact that expansins are involved in cell wall expansion, in an attempt for survival and growth in response to the downy mildew fungus, it can be postulated that the susceptible seedlings can express expansins. Caffeoyl-CoA O-methyltransferases (CCoAOMTs) were expressed only in the susceptible library which can be ascribed to the attempt for lignification in susceptible inoculated seedlings in hypersensitive response.

ATPases, esterase containing lipase domain were expressed in both inoculated resistant and inoculated susceptible library with increased expression in resistant inoculated and this can be corroborated with earlier findings of Zhu *et al*., (2009) and Barakat *et al*., (2009). It is possible that these proteins might be involved in the transport or detoxification of a *Sclerospora graminicola* derived metabolite with the transport of metabolic defense compounds.

Genes for calcium and calcium binding proteins like calmodulin, calnexin were highly expressed in resistant inoculated than susceptible inoculated library. The results are in agreement with the results of Vadassery and Oelmuller, (2009). This can be endorsed by the fact that in pearl millet downy mildew interactions, receptor mediated cytoplasmic calcium elevations could induce defense genes via the activation of ion fluxes at the plasma membrane, an oxidative burst and MAPK activation. The results are also in agreement with the results of Song, (1995) and Feuillet, (1997), where the R gene class containing rice *Xa-21* and wheat *Lr10* encoded receptor-like kinases with an extracellular serine/threonine kinase domain.

V SUMMARY AND CONCLUSION

The present work entitled “Comparative transcriptome analysis from resistant and susceptible pearl millet (*Pennisetum glaucum* L.) genotypes in response to downy mildew (*Sclerospora graminicola* Sacc.) infection” was carried out with an aim:

1. To find out the candidate genes responsible for resistance in pearl millet against downy mildew.
2. Transcriptome comparison between infected and control tissues within susceptible and resistant genotypes.
3. Transcriptome comparison between infected and control tissues of susceptible and resistant pearl millet genotypes.

Seedlings of both resistant (J2290) and susceptible (7042S) genotypes of pearl millet were grown in artificial epiphytotic conditions. Morphological observation through hypersensitive response and biochemical readings through peroxidase activity were coherent for pearl millet-downy mildew pathogenesis.

The libraries were clonally amplified through emulsion PCR and amplified fragments were sequenced in the 454 GS FLX Pyrosequencer. 34 Mb data was generated by 454 pyrosequencing for all the four multiplexed genotypes with a total of 134788 raw reads (34890767 bases). The maximum read length was 604 bases with an average read length of 258 bases. The reads utilized for generating unigenes for all the four libraries were 32596, 46272, 21606, 23135 and the number of unigenes generated were 2139, 2046, 1425 and 694 respectively.

The analysis of sequencing reads were bifurcated to three tiers which included candidate genes involved in resistance in pearl millet

against downy mildew infection, transcripts specifically and differentially expressed in resistant and susceptible inoculated and control; within the resistant and susceptible inoculated and control libraries.

Specific and differential expression of genes in response to downy mildew infection was observed in the genotypes. Overall, it can be concluded that the resistant and susceptible genotypes responded to downy mildew infection by activating resistance responses. The plants try first to wedge the progression of the pathogen by increasing the expression of hydrolases, lignin synthesis and cell death, culminating in the form of hypersensitive response. The infection is also sensed by various kinases like MAPK, serine/ threonine kinases, shaggy kinases mediated through various secondary messengers like Ca^{2+} which activated other transcriptions factors such as Myb, F-box, DEAD box, heat shock transcription factors, DREB, creb and WRKY, which in turn induce the expression of defense related enzymes from the phenylpropanoid, shikimate pathway and other pathways that are involved in resistance to pathogens. The other category of genes that are actively involved in plant resistance to the pathogen, encoded proteins involved in lignin biosynthesis such as Caffeoyl-CoA O-methyltransferases, cytochrome P 450, lipases, isochorismate synthase, S-adenosylmethionine synthase. Among genes in resistance pathogenesis related proteins, specific genes like *mlo, edr,* stress enhanced proteins, *Avr9 cf* proteins were bestowed in providing resistance in pearl millet against downy mildew infection. The sequences generated from both the downy mildew resistant and susceptible libraries covered various biological processes and molecular functions indicating that 454 sequencing constitutes a powerful tool for sequencing the transcriptome.

The current study helped to catalyze the novel purpose of transcriptomic data of pearl millet against downy mildew for the first time and could provide impetus for furtherance of research in the pearl millet-downy mildew infection.

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Appendix

Sequence distribution of unigenes identified in resistant inoculated library

Sequence Name	Sequence Description	Min. eValue	Mean Similarity	GOs	Enzyme Codes
Contig1	protein kinase	7.97E-43	90.40%	7	EC:2.7.10.0; EC:2.7.11.0
Contig102	shaggy-related protein kinase eta	4.79E-64	99.35%	6	EC:2.7.11.0
Contig118	dihydrodipicolinate reductase	1.98E-09	95.20%	6	EC:1.3.1.26
Contig121	fiddlehead-like protein	1.16E-76	93.95%	12	
Contig15	aaa-type atpase family protein	6.18E-27	89.75%	2	EC:3.6.1.15
Contig150	oxygen-evolving enhancer protein 1	4.39E-73	96.00%	6	
Contig214	gata zinc finger family protein	8.05E-35	94.90%	7	
Contig251	xyloglucan endotransglycosylase	3.32E-20	81.10%	7	EC:2.4.1.207; EC:3.2.1.0
Contig278	fkbp12-interacting protein of 37	4.23E-28	84.36%	1	
Contig294	phenylalanine ammonia-lyase	9.18E-31	100.00%	9	EC:4.3.1.5
Contig299	ankyrin repeat domain-containing protein 2	1.85E-31	98.45%	2	
Contig312	cathepsin a	3.77E-08	71.78%	3	EC:3.4.22.0
Contig327	heat shock protein 70	1.63E-19	100.00%	2	
Contig331	allene oxide synthase	1.19E-38	84.50%	14	EC:4.2.1.92
Contig361	gast1 protein precursor	5.49E-31	87.25%	1	
Contig366	beta-glucosidase isozyme 2 precursor	3.05E-34	86.00%	6	EC:3.2.1.118
Contig367	succinyl- ligase	5.60E-28	96.55%	9	EC:2.3.3.8; EC:6.2.1.5
Contig424	diacylglycerol kinase	3.94E-21	92.10%	4	EC:2.7.1.107
Contig435	ring domain containing protein	3.30E-28	79.18%	2	
Contig438	serine threonine protein kinase	3.74E-32	93.40%	5	EC:2.7.112.1
Contig455	esterase lipase domain-containing protein	8.58E-29	84.60%	3	EC:1.4.3.5
Contig456	elmo domain-containing protein 2	1.58E-14	94.55%	2	
Contig458	class iii peroxidase	5.51E-31	87.10%	9	EC:1.11.1.7
Contig461	lipoxygenase 2	2.56E-33	89.95%	9	EC:1.13.11.12
Contig470	transcription factor	3.04E-18	85.25%	3	
Contig497	ubiquitin-conjugating enzyme e2 7	2.77E-13	99.10%	5	EC:6.3.2.19
Contig507	serine threonine-protein kinase	9.88E-26	96.30%	7	EC:2.7.11.0
Contig53	rav-like protein	3.05E-28	85.10%	7	
Contig68	ferredoxin-nadp oxidoreductase	2.81E-19	92.70%	14	EC:1.6.99.1;

					EC:1.18.1.2
Contig75	esterase precursor	1.71E-77	81.30%	4	
Contig80	glutamate dehydrogenase	2.15E-17	99.25%	10	EC:1.4.1.3; EC:1.4.1.2
GWE1CFD01A1ILK	kda proline-rich protein	1.49E-15	90.80%	4	
GWE1CFD01A2EBF	phosphatidylinositol 3- and 4-kinase family protein	3.48E-56	93.05%	2	EC:2.7.1.0
GWE1CFD01A2SL3	s-adenosyl-l-homocysteine hydrolase	2.48E-60	91.10%	5	EC:3.3.1.1
GWE1CFD01A3QZR	glycosyltransferase belonging to cazy family gt61	5.49E-31	69.67%	2	
GWE1CFD01A42PH	cbl-interacting protein kinase	1.30E-40	88.55%	6	EC:2.7.11.0
GWE1CFD01A4PLF	glutamate decarboxylase	4.68E-65	93.60%	10	EC:4.1.1.15; EC:4.1.1.0
GWE1CFD01A6B93	gata transcription factor 9	3.96E-26	80.13%	7	
GWE1CFD01A8TKO	calnexin 1	6.75E-21	93.45%	13	
GWE1CFD01AJSFI	autoinhibited h+ atpase	4.62E-22	99.10%	14	EC:3.6.3.6
GWE1CFD01AK6WN	glycosyltransferase	7.51E-40	86.30%	8	EC:1.13.11.0; EC:1.14.11.19
GWE1CFD01AK7R8	pyruvate kinase	1.48E-36	96.70%	10	EC:2.7.1.40
GWE1CFD01AKKBT	transcription factor apfi	3.62E-11	84.20%	1	
GWE1CFD01AO7TK	Catalase	1.01E-61	87.80%	11	EC:1.11.1.6
GWE1CFD01APK2T	crm family member 3	9.91E-17	82.25%	1	
GWE1CFD01AQ2WA	peroxidase	5.71E-33	71.95%	9	EC:1.11.1.7
GWE1CFD01AQE15	aspartate kinase-homoserine dehydrogenase	5.54E-19	91.05%	15	EC:1.1.1.3; EC:2.7.2.4
GWE1CFD01AQFLS	zinc-finger protein wzf1	2.38E-10	67.71%	13	
GWE1CFD01ARKNW	serine esterase family protein	1.58E-30	92.05%	1	
GWE1CFD01ARR6Y	esterase lipase thioesterase family protein	2.68E-56	79.60%	1	
GWE1CFD01AU0F0	fyve finger-containing phosphoinositide kinase-like	2.31E-20	83.00%	3	EC:2.7.1.68
GWE1CFD01AUDCF	superoxide dismutase	8.40E-22	91.00%	3	
GWE1CFD01AVE2C	peroxidase family expressed	1.04E-26	84.85%	9	EC:1.11.1.7
GWE1CFD01AWML2	cytochrome p450 like_tbp	3.66E-43	91.27%	2	
GWE1CFD01AYQLN	thionin-like peptide	2.00E-09	71.11%	1	
GWE1CFD01AZ1P4	acid phosphatase	1.09E-55	80.50%	5	EC:3.1.3.2
GWE1CFD01B080K	heat shock protein	7.19E-19	96.83%	1	

GWE1CFD01B0C0P	glyceraldehyde-3-phosphate dehydrogenase	3.43E-40	95.05%	6	EC:1.2.1.12
GWE1CFD01B20FE	acetylornithine aminotransferase	1.93E-44	89.45%	17	EC:2.6.1.11
GWE1CFD01B3WBH	stress enhanced protein 1	3.23E-27	79.30%	8	
GWE1CFD01B4KQN	ethylene overproducer-like 1	4.76E-21	94.80%	2	
GWE1CFD01B5KS3	gsdl-motif lipase	1.81E-34	75.05%	3	
GWE1CFD01B763W	salt-inducible protein homolog	4.24E-20	90.85%	2	
GWE1CFD01B7K92	s-adenosylmethionine synthetase	8.70E-48	100.00%	5	EC:2.5.1.6
GWE1CFD01B8OFT	glucan endo -beta-glucosidase 3 precursor	1.59E-51	76.50%	1	
GWE1CFD01BB1IY	thioredoxin f	6.84E-16	80.10%	11	
GWE1CFD01BHLX8	c-repeat binding factor 6	2.29E-13	73.80%	6	
GWE1CFD01BKJ23	kinase-like protein	1.72E-28	95.45%	10	EC:2.7.11.0
GWE1CFD01BKN5X	ring finger 1	3.39E-30	84.00%	2	
GWE1CFD01BKVIC	abc transporter atpase	1.41E-38	91.90%	8	
GWE1CFD01BL0QK	ef hand family expressed	1.95E-49	79.65%	2	
GWE1CFD01BR292	wd-40 repeat	2.04E-26	95.94%	1	
GWE1CFD01BRSCG	nbs-lrr disease resistance protein family-1	3.45E-07	63.00%	1	
GWE1CFD01BSZNJ	glutathione s-transferase	1.26E-11	83.92%	7	EC:2.5.1.18
GWE1CFD01BU0D0	f-box protein	5.23E-26	83.50%	2	
GWE1CFD01BU1H2	aspartic proteinase nepenthesin-1	2.10E-36	77.85%	2	EC:3.4.23.0
GWE1CFD01BU1HP	zn- - containing protein	1.97E-30	65.83%	2	
GWE1CFD01BUUVH	peroxidase 1	9.62E-20	80.65%	9	EC:1.11.1.7
GWE1CFD01CBD6Y	chaperone protein	1.18E-53	87.65%	6	EC:1.3.1.74
GWE1CFD01CCNJO	ubiquitin-conjugating enzyme e2-21 kda 1	4.66E-08	100.00%	3	EC:6.3.2.19
GWE1CFD01CHB3Z	calmodulin-domain protein kinase cdpk isoform 5	1.84E-38	95.30%	9	EC:2.7.11.17; EC:2.7.10.0
GWE1CFD01CHSLU	ferritin 1a	5.30E-26	96.50%	7	EC:1.16.3.1
GZL4SJN01A4KU4	abc transporter related protein	4.71E-27	86.75%	5	EC:3.6.3.30
GZL4SJN01BC8Y9	4-coumarate coenzyme a ligase	1.06E-34	95.95%	6	EC:6.2.1.12
GZL4SJN01BCBVR	e1a creb-binding protein	1.86E-31	91.65%	11	
GZL4SJN01BEP53	wd repeat protein	2.55E-33	85.50%	2	
GZL4SJN01BZWC4	serine threonine protein kinase	5.88E-30	97.90%	6	EC:2.7.11.0
GZL4SJN01C84CE	cellulose synthase	1.49E-28	100.00%	9	EC:2.4.1.12
GZL4SJN01CBAF7	npk1-related protein kinase-like protein	7.22E-23	85.90%	4	
GZL4SJN01D3VOT	avr9 cf-9 rapidly elicited protein 231	4.96E-24	85.90%	2	

GZL4SJN01D9KQP	endo- -beta-glucanase	8.31E-24	96.20%	10	EC:3.2.1.4
GZL4SJN01EYRKV	catalase 2	1.08E-15	81.30%	32	EC:1.11.1.6
GZL4SJN02F4KXN	chalcone synthase	4.42E-17	92.95%	4	EC:2.3.1.74
GZL4SJN02F3OIN	clathrin adaptor complexes medium subunit family protein	1.19E-25	96.00%	5	
GZL4SJN02F4ODL	yt521-b-like family expressed	1.66E-32	93.35%	1	
GZL4SJN02F5AVW	delta-1-pyrroline-5-carboxylate synthetase	1.52E-25	94.25%	10	EC:2.7.2.11; EC:1.2.1.41
GZL4SJN02FK33F	Lipoprotein	4.89E-17	82.90%	2	
GZL4SJN02FKVVQ	cytochrome p450	4.14E-23	86.55%	7	
GZL4SJN02FS0SG	fiddlehead-like protein	1.50E-33	90.45%	4	
GZL4SJN02G9HZB	heat shock protein 90	4.19E-15	100.00%	4	
GZL4SJN02GKPYU	serine carboxypeptidase 1 precursor	1.22E-26	89.80%	3	EC:3.4.16.0
GZL4SJN02GLGHB	argininosuccinate synthase	3.88E-29	93.70%	7	EC:6.3.4.5
GZL4SJN02GVSD6	galactosyltransferase family protein	1.02E-29	90.85%	7	
GZL4SJN02GZ0Y6	ethylene-inducible protein	2.51E-28	95.85%	3	
GZL4SJN02H742F	ubiquitin ligase protein cop1	9.86E-33	96.05%	14	EC:6.3.2.19
GZL4SJN02H8CU7	Chitinase	4.85E-32	94.60%	6	EC:3.2.1.14
GZL4SJN02H9SDM	serine hydroxymethyltransferase	1.14E-36	97.20%	9	EC:2.1.2.1
GZL4SJN02HGIR3	aspartate kinase	2.21E-24	96.70%	9	EC:2.7.2.4
GZL4SJN02HTAAR	transcriptional regulatory protein	2.32E-24	77.40%	7	EC:3.6.1.15
GZL4SJN02I054Y	dre binding factor 2	1.39E-26	79.80%	6	
GZL4SJN02I9GUI	senescence-induced receptor-like serine threonine kinase -like	3.21E-15	97.14%	2	
GZL4SJN02ICEW6	inositol polyphosphate 2-kinase	3.97E-26	89.00%	2	EC:2.7.1.158
GZL4SJN02ICNR0	Enolase	5.69E-33	98.00%	9	EC:4.2.1.11
GZL4SJN02IF0NX	aspartic Proteinase	1.26E-27	91.75%	5	EC:3.4.23.0
GZL4SJN02IHQJ9	dehydration-responsive element binding protein 3	4.10E-15	90.55%	6	
GZL4SJN02IKFT7	tpst_arath ame: full=protein-tyrosine sulfotransferase ame: full=tyrosylprotein sulfotransferase flags: precursor	2.60E-33	84.29%	2	EC:2.8.2.0
GZL4SJN02IM3QY	myb-like dna-binding shaqkyf class family protein	1.09E-15	96.60%	4	
GZL4SJN02IRW80	dihydrofolate reductase-thymidylate synthase	3.00E-21	92.10%	10	EC:1.5.1.3; EC:2.1.1.45
GZL4SJN02IUPR0	alpha beta fold family expressed	1.95E-20	63.16%	2	
GZL4SJN02J1ODI	aminotransferase class iv family protein	2.10E-35	90.80%	9	EC:2.6.1.42
GZL4SJN02J46IB	heat shock cognate 70 kda expressed	2.39E-26	98.20%	2	

GZL4SJN02J4LMA	phosphatidylinositol kinase	5.04E-24	86.05%	1	
GZL4SJN02JCJH3	dihydrolipoamide dehydrogenase precursor	9.03E-26	94.15%	16	EC:1.8.1.4
GZL4SJN02JFJVE	dnaj heat shock n-terminal domain-containing protein	3.09E-16	92.17%	2	
GZL4SJN02JOZRA	pathogenesis-related protein 10	4.73E-19	67.80%	2	
GZL4SJN02JWCI6	chy and ctchy and ring-type zinc finger protein	2.24E-32	94.80%	3	
GZL4SJN02JWWYT	phosphoglycerate kinase	1.73E-21	96.00%	7	EC:2.7.2.3
GZL4SJN02JZUDP	leucine-rich repeat protein	3.37E-33	91.05%	6	

Sequence distribution of unigenes identified in resistant control genotype

Sequence Name	Sequence Description	Min. eValue	Mean Similarity	GOs	Enzyme Codes
Contig100	myb transcription factor	4.39E-09	96.00%	3	
Contig11	beta-glucosidase isozyme 2 precursor	5.13E-37	75.75%	6	EC:3.2.1.0
Contig115	arm repeat protein	3.86E-53	74.00%	6	EC:6.3.2.19
Contig123	transcription factor	4.92E-58	89.50%	1	
Contig137	dead box rna helicases	2.24E-37	84.65%	3	
Contig178	abc transporter family protein	2.41E-43	95.10%	6	
Contig189	aldehyde oxidase	9.53E-60	80.85%	9	EC:1.2.3.1
Contig194	chalcone synthase	1.27E-49	79.70%	6	EC:2.3.1.74
Contig226	adp-ribosylation factor	2.30E-21	100.00%	10	
Contig255	h^+atpase 2	6.97E-23	84.90%	7	EC:3.6.3.6; EC:3.6.1.3
Contig259	glycerol-3-phosphate acyltransferase	7.20E-07	97.00%	3	
Contig283	xyloglucan endotransglycosylase	3.51E-14	94.90%	6	EC:3.2.1.0; EC:2.4.1.207
Contig308	3-ketoacyl- thiolase	3.28E-28	91.70%	9	EC:2.3.1.16
Contig353	calnexin 1	9.10E-18	94.20%	11	
Contig358	polyubiquitin containing 7 ubiquitin monomers	4.47E-25	99.20%	1	
Contig363	*mlo*-like protein 4	6.75E-18	93.65%	6	
Contig371	5-enolpyruvylshikimate-3-phosphate synthase	2.57E-25	97.40%	6	EC:2.5.1.19
Contig387	cell wall-associated hydrolase	3.49E-88	91.35%	3	
Contig402	tpa: class iii peroxidase 65 precursor	8.07E-36	91.20%	7	EC:1.11.1.7

Contig48	cbl-interacting protein kinase	1.27E-17	88.42%	4	EC:2.7.11.0
Contig5	early-responsive to dehydration stress-related protein	2.46E-27	82.70%	2	
Contig50	Aquaporins	9.11E-27	95.00%	4	
Contig54	cellulose synthase	2.26E-55	99.75%	5	EC:2.4.1.12
Contig57	beta purothionin	6.82E-30	62.30%	1	
Contig67	serine-threonine kinase	2.63E-109	92.80%	5	EC:2.7.11.0
Contig75	adp-ribosylation factor 1	4.02E-66	98.70%	8	
Contig8	nifu-like n-terminal domain-containing protein	1.46E-12	98.65%	5	
Contig86	fatty acid hydroperoxide lyase	3.96E-45	77.60%	4	
Contig9	tubulin alpha-3 chain	2.91E-24	99.15%	7	EC:3.6.5.1; EC:3.6.5.2; EC:3.6.5.3; EC:3.6.5.4
Contig91	hydroxyproline-rich glycoprotein family protein	4.19E-39	77.40%	1	
GWE1CFD01A2AAD	ribonuclease 1	4.93E-48	67.75%	10	EC:3.1.27.1
GWE1CFD01A3DT5	serine threonine-specific kinase like protein	1.27E-19	91.90%	7	EC:2.7.11.0
GWE1CFD01A3JT0	heat shock protein 90	8.28E-24	86.55%	4	
GWE1CFD01A3MHD	dreg-2 like protein	4.89E-48	88.47%	3	EC:3.1.3.18
GWE1CFD01A4YAU	rubredoxin family expressed	1.10E-31	87.25%	3	
GWE1CFD01A66NE	chitin-inducible gibberellin-responsive protein	1.42E-15	82.89%	1	
GWE1CFD01A98EA	nb-arc domain containing expressed	8.38E-40	87.80%	1	
GWE1CFD01A9U4W	alpha tubulin	2.30E-13	100.00%	8	EC:3.6.5.1; EC:3.6.5.2; EC:3.6.5.3; EC:3.6.5.4
GWE1CFD01AJMWW	glutathione s-transferase	3.93E-21	80.45%	2	EC:2.5.1.18
GWE1CFD01AJXFD	transducin family protein wd-40 repeat family protein	1.13E-23	80.25%	1	
GWE1CFD01ANHJ5	endochitinase a2 precursor	1.30E-16	95.00%	4	EC:3.2.1.14
GWE1CFD01AOWTJ	beta-galactosidase	7.22E-23	84.30%	4	EC:3.2.1.23
GWE1CFD01AP5WK	abc1 family protein	9.33E-60	84.00%	4	
GWE1CFD01AQTZ6	formin-like protein	8.75E-38	86.10%	8	
GWE1CFD01ARP51	flavonoid 7-o-methyltransferase	3.00E-21	60.95%	1	
GWE1CFD01AU85V	serine threonine-protein kinase nak	3.21E-34	90.05%	4	EC:2.7.11.0
GWE1CFD01AVLZ3	ankyrin-like protein	1.58E-14	91.06%	1	
GWE1CFD01AVWR	sf16 protein	6.13E-11	72.20%	1	

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GWE1CFD01AW7RA	arf gtpase-activating domain-containing protein	3.00E-48	83.30%	8	
GWE1CFD01AWD5M	leucine carboxyl methyltransferase family protein	1.17E-17	89.07%	1	EC:2.1.1.0
GWE1CFD01AWQ5P	cytochrome p450	7.14E-44	92.80%	5	
GWE1CFD01AX0MM	shaggy-related protein kinase eta	5.57E-37	76.45%	10	
GWE1CFD01AXWYM	wd-repeat membrane protein	2.32E-13	97.19%	3	
GWE1CFD01AYI2S	phd finger family protein	9.75E-17	81.85%	3	
GWE1CFD01AZXYC	arginine decarboxylase	3.20E-68	86.45%	5	EC:4.1.1.19
GWE1CFD01B1GOX	aldehyde dehydrogenase family 7 member a1	1.29E-49	90.50%	9	EC:1.2.1.3
GWE1CFD01B3GGV	aminotransferase family protein	1.14E-19	81.25%	9	EC:2.6.1.5; EC:2.6.1.80; EC:4.4.1.14
GWE1CFD01B3HF9	catalytic hydrolase	1.73E-37	90.55%	2	
GWE1CFD01B4LYF	serine-threonine protein plant-	4.25E-20	62.15%	2	
GWE1CFD01B4RI1	transcription factor qsh-1	5.05E-24	98.35%	6	
GWE1CFD01B5ELB	5-methyltetrahydropteroyltriglutamate-homocysteine expressed	4.86E-24	96.20%	3	EC:2.1.1.14
GWE1CFD01B5SBE	enhanced disease resistance 1	5.87E-22	82.88%	6	EC:2.7.10.0; EC:2.7.11.0
GWE1CFD01B6J9U	allene oxide synthase	5.47E-57	83.85%	13	EC:4.2.1.92
GWE1CFD01B713B	cathepsin b	2.20E-40	87.30%	4	EC:3.4.22.0
GWE1CFD01B79LH	ethylene response factor	1.55E-22	74.30%	5	
GWE1CFD01B8337	flavin reductase like domain protein	1.79E-35	79.85%	3	
GWE1CFD01B8H1N	lysine-specific histone demethylase 1	5.96E-43	92.05%	6	EC:1.5.3.11; EC:1.4.3.4; EC:1.4.3.6; EC:2.1.1.0
GWE1CFD01B9R04	knotted1 homeodomain protein	5.99E-14	96.00%	5	
GWE1CFD01BA3S0	kinesin-related protein	8.95E-34	85.85%	8	
GWE1CFD01BA9OK	tetratricopeptide repeat	1.51E-33	74.15%	2	
GWE1CFD01BBJDQ	Catalase	5.70E-33	81.75%	7	EC:1.11.1.6
GWE1CFD01BBJHX	phenylalanine ammonia-lyase	7.39E-30	97.70%	6	EC:4.3.1.5
GWE1CFD01BFNV9	glyoxal oxidase	8.85E-10	76.20%	1	
GWE1CFD01BHFN2	protein kinase apk1a	6.97E-18	89.00%	3	EC:2.7.11.0
GWE1CFD01BHU09	glycolate oxidase	1.21E-56	83.70%	4	

GWE1CFD01BNJL4	zinc finger	7.73E-21	69.25%	1	
GWE1CFD01BO942	betaine aldehyde dehydrogenase-like	2.50E-34	85.45%	4	
GWE1CFD01BQ3TZ	rxo1 disease resistance protein	4.07E-10	75.25%	4	
GWE1CFD01BQBRV	dehydroascorbate reductase	3.64E-36	83.95%	1	
GWE1CFD01BRBE2	tetratricopeptide-like helical	4.28E-47	67.60%	1	
GWE1CFD01BRHBH	bel1-type homeodomain protein	9.97E-53	95.20%	5	
GWE1CFD01BT1B5	mynd finger family expressed	3.34E-47	83.40%	2	EC:2.1.1.0
GWE1CFD01BU5NA	Arginase	1.61E-43	90.65%	8	EC:3.5.3.1
GWE1CFD01BVOHK	myb family transcription factor-related protein	9.26E-59	88.55%	4	
GWE1CFD01BW2A4	calcium-dependent protein kinase	1.23E-29	97.25%	4	EC:2.7.11.0
GWE1CFD01BX4CH	kelch repeat-containing protein	3.17E-15	83.42%	2	
GWE1CFD01BXH9S	fmn binding protein	3.58E-19	85.40%	2	
GWE1CFD01BYKQS	cellulose synthase-like d3-glycosyltransferase family 2 protein	2.18E-24	84.45%	4	EC:2.4.1.12
GWE1CFD01BZ842	f-box protein fbl2	1.03E-37	90.45%	4	EC:6.3.2.19
GWE1CFD01BZZH4	leucine-rich repeat protein	1.69E-25	93.95%	5	
GWE1CFD01CBY6G	thioredoxin h-type	2.94E-08	81.00%	4	
GWE1CFD01CC77C	argonaute protein group	6.83E-48	90.65%	9	
GWE1CFD01CEPB6	amp dependent	9.76E-09	92.62%	3	
GWE1CFD01CG1O5	cbs domain-containing protein	4.95E-32	91.55%	3	
GWE1CFD01CGZV5	kinase family protein	1.20E-43	86.70%	4	EC:2.7.11.0
GWE1CFD01CIUTP	dnaj heat shock n-terminal domain-containing expressed	4.97E-24	92.00%	2	
GWE1CFD01CIW11	serine hydroxymethyltransferase	3.70E-19	96.25%	7	EC:2.1.2.1
GZL4SJN01A3L0R	glutathione s-transferase gstu6	3.53E-22	85.46%	2	
GZL4SJN01A9UMX	beta-adaptin-like protein a	2.77E-27	75.67%	5	
GZL4SJN01AD69C	hydroxymethyltransferase-like protein	8.41E-16	92.00%	5	EC:2.1.2.1
GZL4SJN01B61AM	jacalin-like lectin domain containing expressed	5.42E-15	75.31%	1	
GZL4SJN01B6CIJ	tbc domain containing protein	6.11E-27	85.05%	3	
GZL4SJN01BN781	thiamine biosynthesis protein	6.52E-28	99.10%	5	
GZL4SJN01BOBYY	Peroxidase	2.45E-23	87.00%	7	EC:1.11.1.7
GZL4SJN01BSFVH	glyceraldehyde-3-phosphate dehydrogenase	1.03E-21	100.00%	5	EC:1.2.1.12
GZL4SJN01BX3XH	cytochrome oxidase subunit 3	1.24E-27	93.95%	6	EC:1.9.3.1
GZL4SJN01CEO1P	zinc finger protein 33b	1.48E-10	65.67%	2	
GZL4SJN01D8XMZ	fumarate reductase	4.02E-16	87.83%	10	EC:1.3.99.1; EC:1.3.5.1
GZL4SJN01D98JM	transcriptional regulator	4.37E-33	66.15%	5	

GZL4SJN01DIKB2	ribonuclease g	4.63E-14	91.85%	8	
GZL4SJN01ETF85	fe^{3+} abc permease	3.01E-29	84.00%	6	
GZL4SJN02F5CYF	zinc finger protein 3-like	1.99E-17	82.85%	8	
GZL4SJN02F9595	cysteine synthase	1.43E-23	95.80%	7	EC:2.5.1.47
GZL4SJN02FKOY4	disease resistance protein family protein	1.61E-19	77.50%	2	
GZL4SJN02GDPPN	casein kinase 2 subunit beta	1.10E-31	97.70%	6	
GZL4SJN02GR74L	wd-40 repeat family	1.50E-20	88.25%	1	
GZL4SJN02GUDHD	pyruvate orthophosphate dikinase	9.68E-28	96.90%	11	EC:2.7.9.1
GZL4SJN02H5IJX	serine threonine protein kinase	3.61E-27	89.00%	5	EC:2.7.10.0; EC:2.7.11.0
GZL4SJN02H5IWX	mitogen activated protein kinase kinase kinase	1.34E-37	92.10%	5	EC:2.7.11.0
GZL4SJN02HWXNB	cytochrome p450 like_tbp	3.08E-35	91.88%	2	
GZL4SJN02HZUVK	c2 domain-containing protein	8.63E-37	94.35%	3	
GZL4SJN02I37VR	Chaperonin	6.95E-31	100.00%	5	
GZL4SJN02I3IC7	glycosyl hydrolase family 3 c terminal domain containing expressed	9.28E-39	93.40%	3	EC:3.2.1.0
GZL4SJN02IJIE7	4-hydroxyphenylacetate 3-monooxygenase	2.83E-40	87.15%	7	EC:1.14.14.0; EC:1.3.99.3; EC:1.14.13.3
GZL4SJN02IO9L7	gtp-binding protein era	1.78E-28	99.25%	8	
GZL4SJN02IU3ME	3-phosphoshikimate 1-carboxyvinyltransferase	7.49E-25	85.75%	3	EC:2.5.1.19
GZL4SJN02IYKLC	dihydroflavonol-4-reductase	2.85E-32	87.45%	3	
GZL4SJN02J2M40	heat shock cognate 70 kda expressed	9.78E-25	98.20%	2	
GZL4SJN02J6RLC	dihydrolipoamide dehydrogenase	1.01E-24	98.90%	6	EC:1.8.1.4

Sequence distribution of unigenes identified in susceptible inoculated genotype

Sequence Name	Sequence Description	Min. eValue	Mean Similarity	GOs	Enzyme Codes
Contig106	oxygen-evolving enhancer protein 1	1.03E-67	93.45%	6	
Contig110	zinc transporter	2.54E-44	92.00%	6	
Contig142	tryptophan synthase beta chain	2.48E-19	82.90%	4	
Contig143	beta-expansin 1a	5.57E-44	91.40%	3	
Contig15	thioredoxin-related protein	4.77E-43	90.55%	2	
Contig151	chitinase 1 precursor	1.16E-09	91.00%	3	EC:1.6.5.3
Contig166	ethylene receptor	2.21E-39	81.50%	10	

Contig172	metallothionein-like protein	8.38E-08	83.00%	1	EC:3.4.23.0
Contig175	glycine-rich protein	1.82E-44	86.88%	1	EC:2.3.1.0
Contig191	rpt2-like protein	2.33E-44	85.65%	3	
Contig217	ribonuclease hi large	2.18E-25	85.50%	1	EC:3.6.3.14
Contig226	kinase family protein	2.53E-12	86.05%	5	
Contig233	wd-40 repeat family	1.96E-25	97.11%	1	EC:3.6.5.3
Contig240	cell wall-associated hydrolase	6.80E-107	90.10%	4	
Contig245	kda proline-rich protein	2.58E-17	85.20%	3	
Contig299	photosystem i p700 apoprotein a1	1.51E-12	100.00%	12	EC:2.4.1.207; EC:3.2.1.0
Contig30	armadillo repeat containing	1.01E-21	88.64%	5	
Contig313	aspartate kinase-homoserine dehydrogenase	2.58E-25	89.70%	11	
Contig320	nadh dehydrogenase subunit 3	1.90E-36	96.15%	9	
Contig38	argininosuccinate synthase	2.16E-19	94.70%	3	
Contig71	immediate-early fungal elicitor protein cmpg1	4.79E-39	82.30%	6	
Contig84	adenine nucleotide translocator	5.93E-22	95.80%	14	
GWE1CFD01A0DNX	cysteine protease 1	1.41E-18	96.85%	4	
GWE1CFD01A0QAY	pentatricopeptide repeat-containing protein	1.74E-16	92.25%	5	
GWE1CFD01A2G8Q	callose synthase 1 catalytic subunit	7.04E-31	98.05%	3	
GWE1CFD01A3WPT	hedgehog-interacting protein	2.95E-21	85.25%	3	
GWE1CFD01A42GG	g2-like myb-family transcription factor	2.70E-30	83.95%	5	
GWE1CFD01A6LJT	c-repeat binding factor 6	2.27E-13	73.80%	4	EC:3.4.17.0; EC:3.2.1.58
GWE1CFD01A8IEY	wrky25 - superfamily of tfs having wrky and zinc finger domains	2.42E-15	94.20%	4	
GWE1CFD01ANKN9	cazy family gt8	6.34E-72	90.50%	3	
GWE1CFD01ANN70	tubby protein	2.46E-31	78.50%	3	
GWE1CFD01ANQCR	allene oxide synthase	2.43E-55	93.50%	13	EC:4.1.1.50
GWE1CFD01AP4EL	beta glucanase	9.19E-15	97.15%	3	
GWE1CFD01APPPN	ap2-domain dre binding factor dbf1	9.97E-25	79.70%	4	
GWE1CFD01ARINV	exoribonuclease r	8.00E-65	97.70%	3	EC:2.7.1.68
GWE1CFD01ARYX9	beta-ig-h3 domain-containing expressed	3.91E-20	95.00%	1	
GWE1CFD01AT0UC	phenylalanine ammonia-lyase	1.15E-33	100.00%	6	EC:2.4.1.0
GWE1CFD01ATVLY	gtp-binding protein	6.22E-36	93.90%	2	
GWE1CFD01AU5NS	regulatory protein	3.61E-35	79.50%	2	
GWE1CFD01AUIA1	ubiquitin-conjugating enzyme e2 16	1.12E-27	97.80%	7	
GWE1CFD01AUWT8	homeodomain leucine zipper protein cphb-5	2.58E-17	85.25%	4	

GWE1CFD01AW3LR	glycogenin expressed	6.76E-51	84.35%	2	
GWE1CFD01AXIC6	wd-40 repeat family	1.67E-32	88.55%	1	EC:3.6.5.3
GWE1CFD01AY82J	aminotransferase y4ub	1.43E-55	96.10%	3	
GWE1CFD01B19YP	homoserine dehydrogenase-like	3.03E-10	89.43%	1	
GWE1CFD01B2M55	fyve finger-containing phosphoinositide	1.85E-14	86.53%	1	
GWE1CFD01B3CVB	threonine endopeptidase	2.22E-39	87.00%	3	
GWE1CFD01B3HCT	ring-h2 finger protein	4.28E-28	59.42%	1	
GWE1CFD01B4KPT	leucine rich repeat family expressed	2.06E-14	93.56%	1	EC:2.3.3.8
GWE1CFD01B6D4Z	biotin synthase	5.96E-38	74.35%	4	
GWE1CFD01B6NK4	expansin-like 3 precursor	9.54E-28	78.85%	4	
GWE1CFD01B8K6U	cytochrome p450 like_tbp	1.48E-30	90.25%	2	
GWE1CFD01B8WL4	ras-related protein rab-18	4.26E-07	81.75%	3	
GWE1CFD01BAQX6	abc transporter	4.03E-31	80.00%	8	
GWE1CFD01BBNEY	cytochrome p450 monooxygenase	9.89E-49	80.35%	3	
GWE1CFD01BCE9F	ribonuclease p family protein	6.19E-27	63.21%	2	
GWE1CFD01BCVK7	cysteine synthase	9.02E-26	70.05%	3	
GWE1CFD01BDEGR	gras family transcription factor containing expressed	7.96E-14	68.30%	1	EC:2.7.1.46; EC:2.7.1.6
GWE1CFD01BE2U3	caffeoyl- 3-o-methyltransferase 1	2.81E-32	83.40%	1	
GWE1CFD01BEIE7	tetratricopeptide repeat protein 1	1.37E-35	89.45%	2	
GWE1CFD01BGNHM	xyloglucan endotransglycosylase	3.05E-90	94.05%	6	
GWE1CFD01BGVN2	peptidase c1a papain family protein	3.88E-69	64.00%	3	EC:3.6.5.3
GWE1CFD01BHGQ0	Catalase	4.92E-52	99.05%	6	
GWE1CFD01BIEYC	protein kinase	4.91E-40	90.60%	4	
GWE1CFD01BL3E2	serine threonine-protein kinase	2.01E-41	89.35%	3	EC:3.4.23.0
GWE1CFD01BLIXT	f-box protein	2.21E-32	89.35%	2	EC:3.2.1.0; EC:2.4.1.207
GWE1CFD01BM7OX	c-x8-c-x5-c-x3-h type zn-finger	1.44E-60	98.65%	3	
GWE1CFD01BONUV	pyruvate kinase	3.13E-63	98.90%	6	EC:1.14.99.7
GWE1CFD01BQL61	superkiller protein 3-like protein	2.58E-17	92.93%	1	
GWE1CFD01BRC0B	ubiquitin-protein ligase	4.43E-17	67.23%	3	EC:3.6.3.14
GWE1CFD01BSBGB	receptor protein kinase	1.73E-53	85.65%	6	EC:3.1.1.0; EC:3.1.1.1
GWE1CFD01BVEEZ	chalcone isomerase-like	3.44E-09	89.00%	2	EC:2.7.11.0
GWE1CFD01BX0FF	o-methyltransferase family expressed	6.18E-27	88.65%	2	

GWE1CFD01CAQNH	zinc finger	3.84E-29	76.33%	2	EC:1.13.11.54
GWE1CFD01CBI2K	glycosyl hydrolase family 1 expressed	2.41E-31	93.75%	7	
GWE1CFD01CBXJ9	ser thr protein phosphatase family	9.36E-19	80.86%	4	
GWE1CFD01CD1DN	strubbelig receptor family 3	1.79E-18	79.90%	8	
GWE1CFD01CDR20	tetratricopeptide repeat-containing protein	4.07E-17	92.75%	4	EC:3.1.26.4; EC:2.7.7.49
GWE1CFD01CH94L	rna helicases	2.22E-32	81.70%	6	
GWE1CFD01CHX1K	Ferredoxin	5.62E-28	92.61%	5	EC:3.6.3.0
GWE1CFD01CIAV2	cellulose synthase-like protein slf1	1.68E-48	88.05%	5	
GZL4SJN02G2UPQ	thaumatin-like protein precursor	1.59E-14	76.83%	1	
GZL4SJN02G43V8	heat shock 70 kda protein	1.84E-31	96.90%	5	EC:3.6.5.1; EC:3.6.5.2; EC:3.6.5.3; EC:3.6.5.4
GZL4SJN02GUBDF	wd repeat	1.45E-31	85.75%	1	
GZL4SJN02HMFBY	ring finger 1	9.78E-17	76.00%	2	EC:3.6.1.15
GZL4SJN02HTXVQ	phosphoglycerate kinase	8.37E-24	87.15%	3	
GZL4SJN02IO8NM	ribosomal protein s14	2.99E-29	90.50%	12	
GZL4SJN02JJGAW	asparagine synthetase	4.36E-36	98.20%	3	

Sequence distribution of unigenes identified in susceptible control genotype

Sequence Name	Sequence Description	Min. eValue	Mean Similarity	GOs	Enzyme Codes
Contig123	abc transporter family protein	1.77E-28	91.35%	1	
Contig128	serine threonine protein kinase	2.67E-22	97.60%	4	EC:2.7.11.0
Contig132	beta purothionin	1.32E-13	76.70%	1	
Contig163	methionine s-methyltransferase	4.77E-27	78.32%	6	EC:2.1.1.12
Contig18	photosystem i p700 apoprotein a1	5.36E-54	100.00%	13	
Contig3	adenylosuccinate synthetase	1.44E-29	95.50%	5	EC:6.3.4.4
Contig33	serine acetyltransferase	4.34E-45	74.45%	1	
Contig35	oxygen-evolving enhancer protein 1	1.87E-57	91.50%	6	
Contig5	cell wall-associated hydrolase	1.95E-91	89.75%	4	
Contig54	dof zinc finger protein mnb1a	1.57E-14	70.80%	2	
GWE1CFD01A00O6	ycf9	5.15E-21	98.10%	6	
GWE1CFD01A5NGR	s-adenosylmethionine synthetase	1.87E-55	99.05%	7	EC:2.5.1.6

GWE1CFD01AP3M1	Zac	1.32E-29	86.00%	7	
GWE1CFD01AQKB1	cytochrome oxidase subunit 1	9.52E-44	100.00%	10	EC:1.9.3.1
GWE1CFD01ASE4N	zinc finger a20 and an1 domains-containing protein	5.44E-31	78.00%	2	
GWE1CFD01B6Z5L	bhlh transcription factor	4.78E-35	74.64%	3	
GWE1CFD01B776B	cytochrome c oxidase subunit 2	6.05E-22	81.50%	10	EC:1.9.3.1
GWE1CFD01B907W	Catalase	4.07E-38	92.95%	6	EC:1.11.1.6
GWE1CFD01B9BXJ	mitogen activated protein kinase kinase	3.90E-24	91.95%	3	EC:2.7.11.0
GWE1CFD01BCL1V	ascorbate peroxidase	4.39E-41	92.40%	4	EC:1.11.1.11
GWE1CFD01BDLKP	udp-glucose 6- expressed	1.55E-22	97.40%	4	EC:1.1.1.22
GWE1CFD01BEM6V	chaperone protein dnaj 49	5.10E-21	88.29%	4	
GWE1CFD01BG8YG	sam domain family protein	6.73E-24	85.35%	3	
GWE1CFD01BGZUQ	inositol-tetrakisphosphate 1-kinase 3	2.95E-29	91.30%	6	EC:2.7.1.134; EC:2.7.1.159
GWE1CFD01BJDF4	domain protein	7.91E-38	73.36%	5	
GWE1CFD01BJFPU	serine threonine kinase receptor precursor-like protein	5.28E-34	78.80%	5	EC:2.7.11.0
GWE1CFD01BKSGW	cbl-interacting serine threonine-protein kinase 1	2.13E-27	94.20%	6	EC:2.7.10.0; EC:2.7.11.0
GWE1CFD01BKU14	chloroplast heat shock protein 70	8.53E-08	89.80%	4	
GWE1CFD01CCRNI	leucine-rich repeat extensin 1	7.01E-51	84.10%	4	
GWE1CFD01CD4QW	beta-glucosidase isozyme 2 precursor	4.63E-43	87.40%	5	EC:3.2.1.0
GWE1CFD01CE7H3	mrp-like abc transporter	3.26E-12	70.94%	2	
GZL4SJN02F1FWF	wd-40 repeat	9.73E-33	93.00%	1	
GZL4SJN02FO5K0	expansin expa11	1.58E-38	96.95%	3	
GZL4SJN02G2OM2	receptor protein kinase	4.45E-25	87.20%	11	EC:2.7.11.0
GZL4SJN02GMPD7	zinc finger (ccch-type)	4.83E-24	86.55%	7	EC:2.7.7.49; EC:3.6.5.1; EC:3.6.5.2; EC:3.6.5.3; EC:3.6.5.4
GZL4SJN02GQPJV	terpene synthase metal binding domain containing expressed	4.97E-32	74.00%	1	
GZL4SJN02HA78G	cystathionine beta-lyase	1.28E-24	82.80%	4	EC:4.4.1.8
GZL4SJN02HDURD	5-enolpyruvylshikimate-3-phosphate synthase	4.11E-23	95.90%	5	EC:2.5.1.19
GZL4SJN02IBOSJ	map kinase	2.72E-33	98.35%	4	EC:2.7.11.24
GZL4SJN02JDFPW	heat shock protein 70	5.10E-29	100.00%	2	
GZL4SJN02JXYSG	proline dehydrogenase delta-1-pyrroline-5-carboxylate dehydrogenase	9.06E-10	76.43%	3	

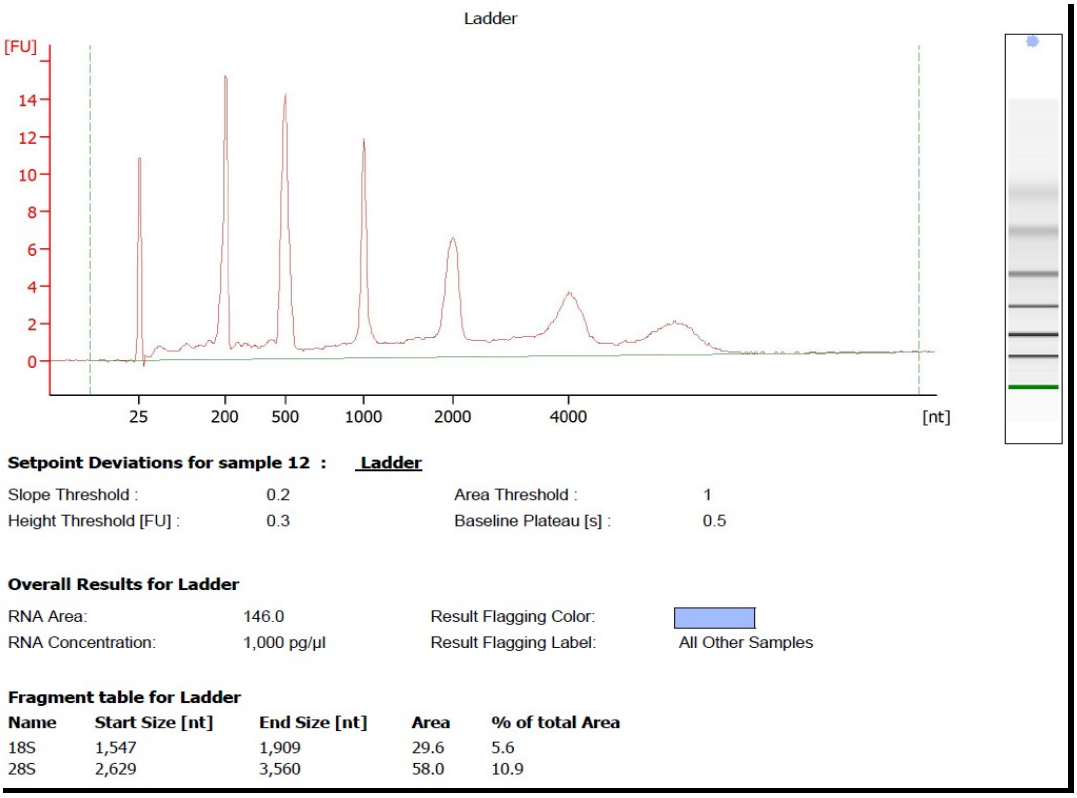
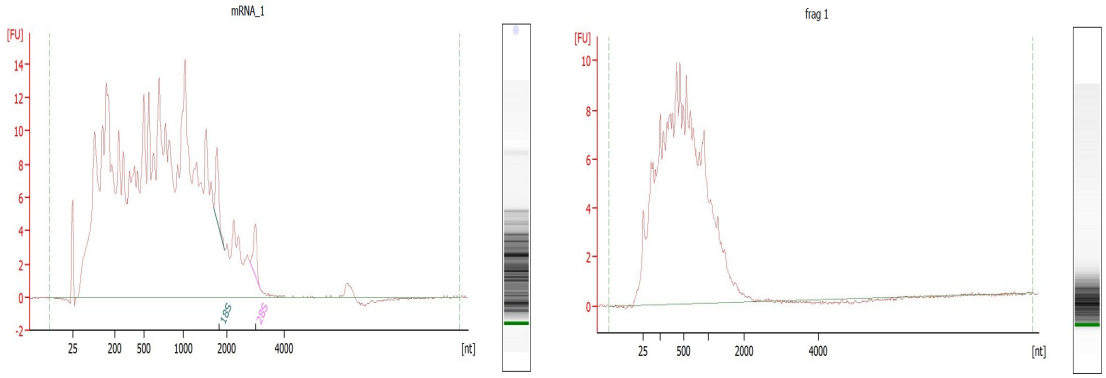


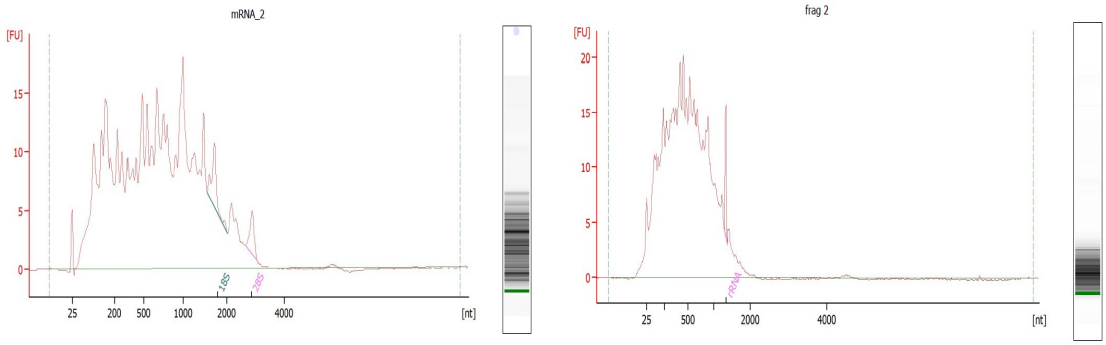
Fig 4.1 Flowgram of RNA pico ladder on Bioanalyzer



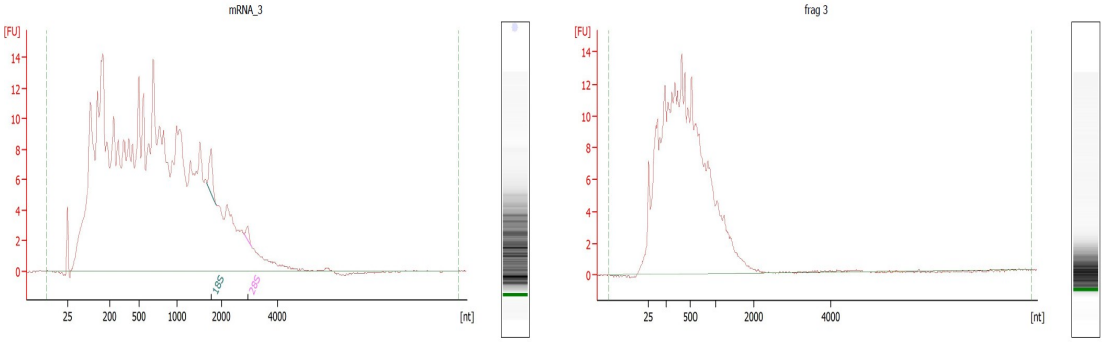
(a) Flowgram of resistant inoculated mRNA before fragmentation and after fragmentation on RNA Pico chip

Fig 4.2 (a) Flowgram of mRNA of pearl millet genotypes before fragmentation

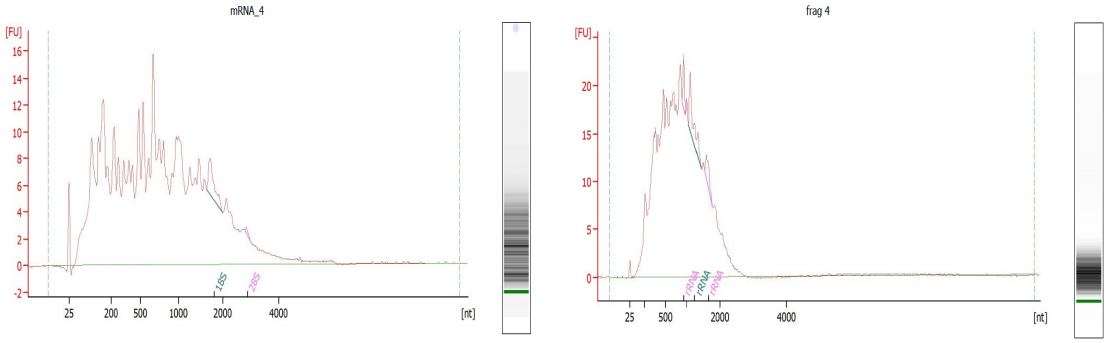
and after fragmentation on RNA Pico chip



(b) Flowgram of resistant control genotype mRNA before fragmentation and after fragmentation on RNA Pico chip



(c) Flowgram of susceptible inoculated genotype mRNA before fragmentation and after fragmentation on RNA Pico chip



(d) Flowgram of susceptible control genotype mRNA before fragmentation and after fragmentation on RNA Pico chip

Fig 4.2 (b-d) Flowgram of mRNA of pearl millet genotypes before fragmentation

and after fragmentation on RNA Pico chip

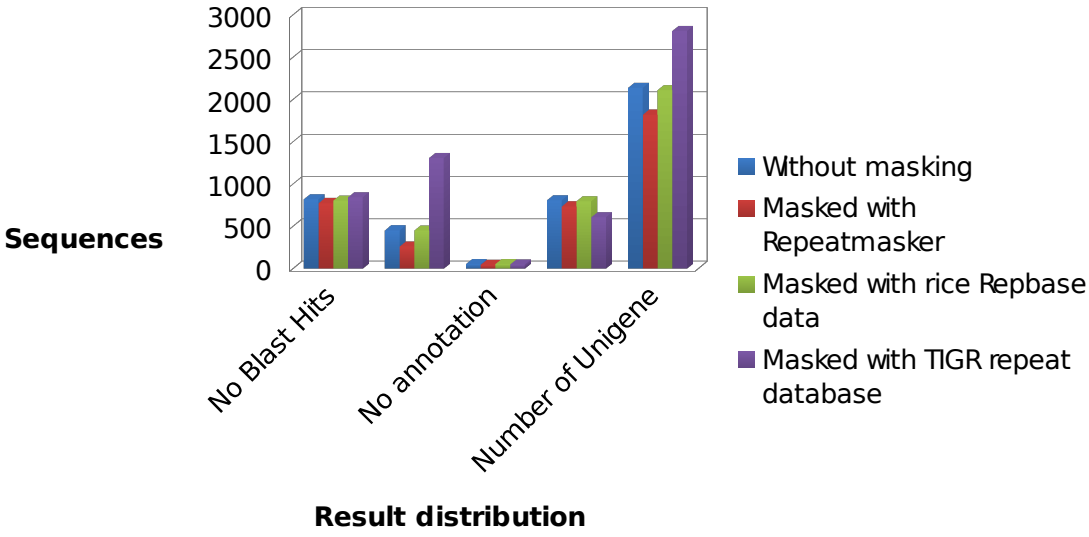


Fig 4.3 Assembly comparison of pearl millet resistant inoculated library sequences with four different parameters

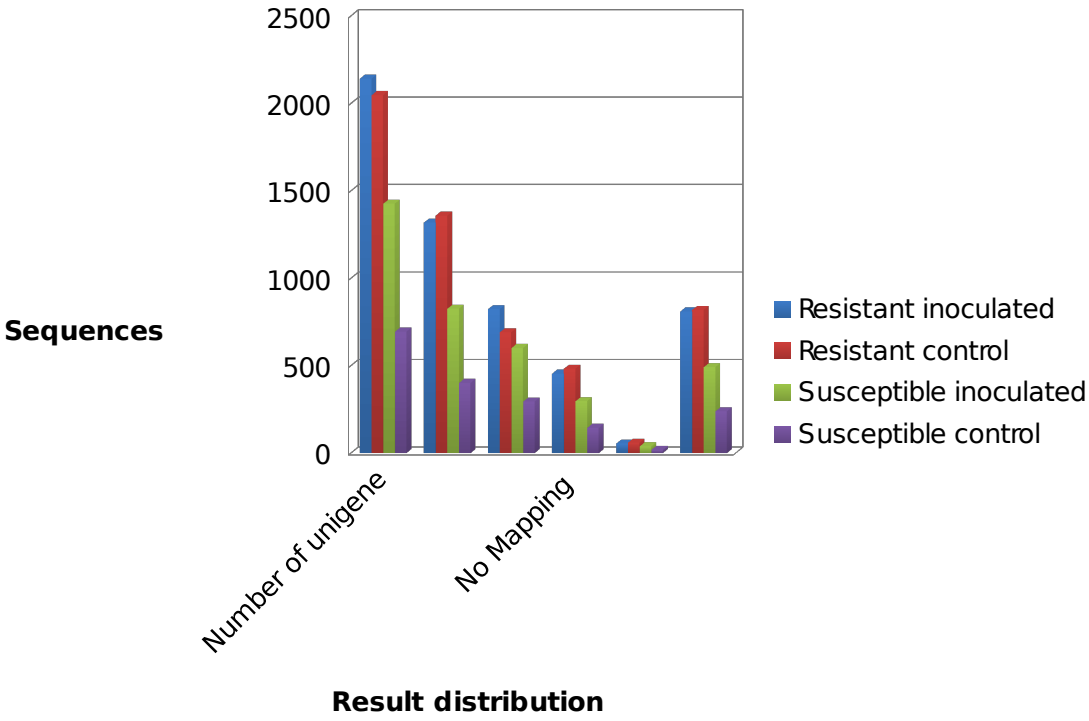


Fig 4.4 Result distribution of pearl millet cDNA library sequences

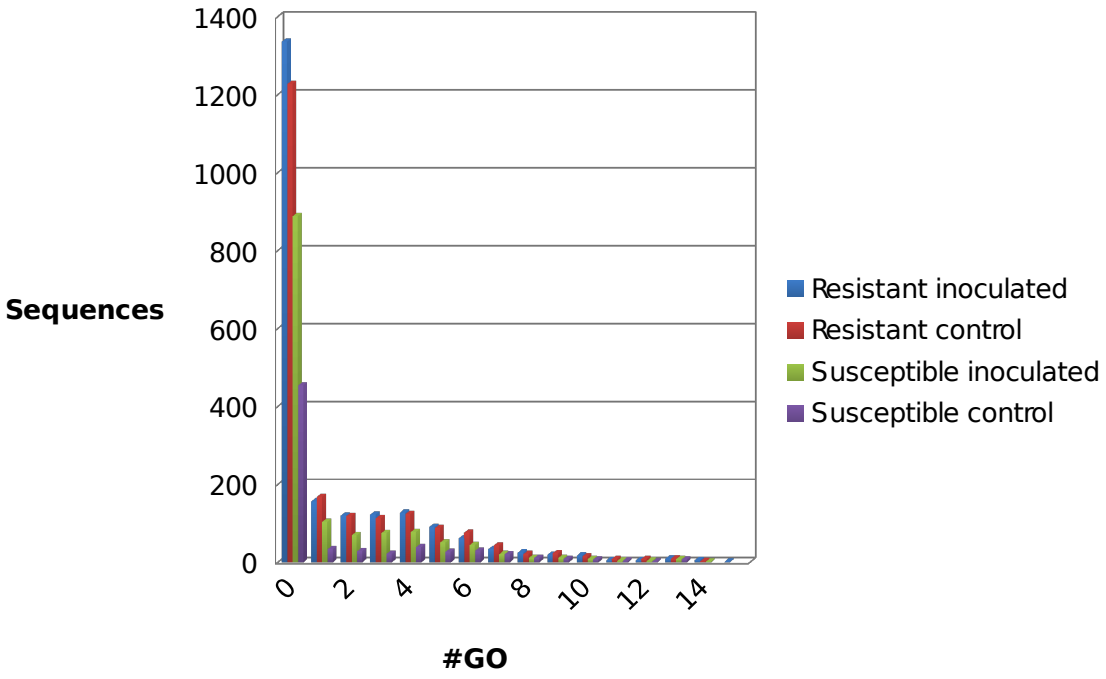


Fig 4.5 Annotation distribution of pearl millet sequences

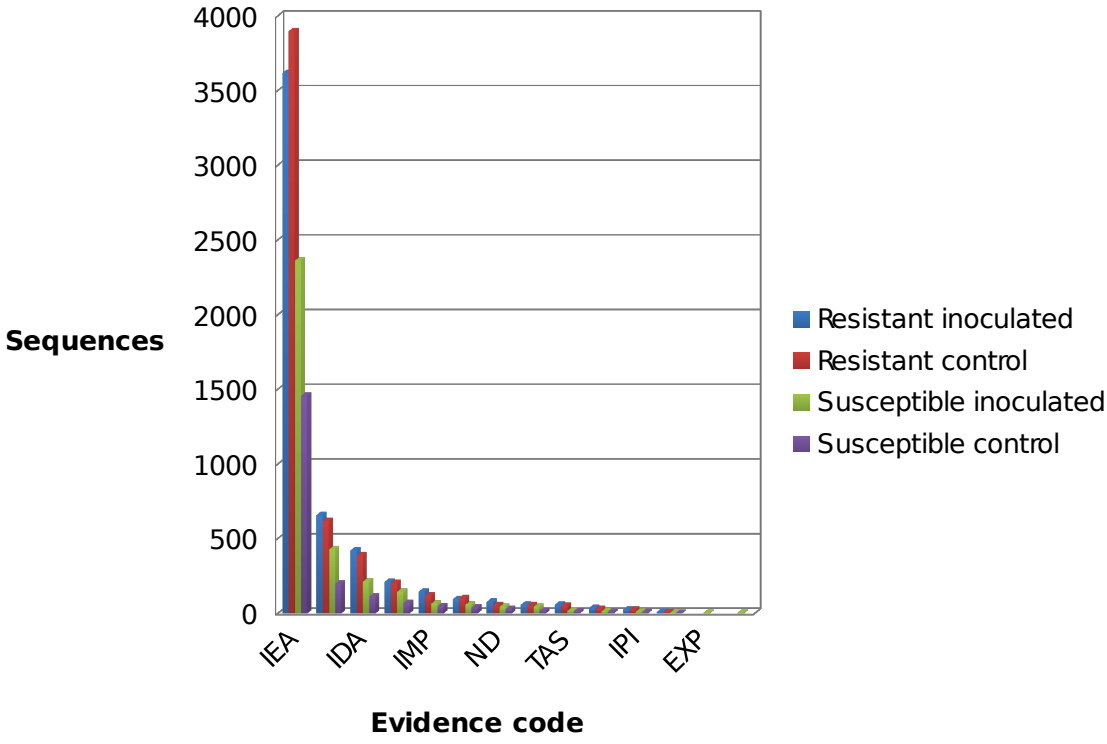


Fig 4.6 Evidence code distribution for pearl millet sequences

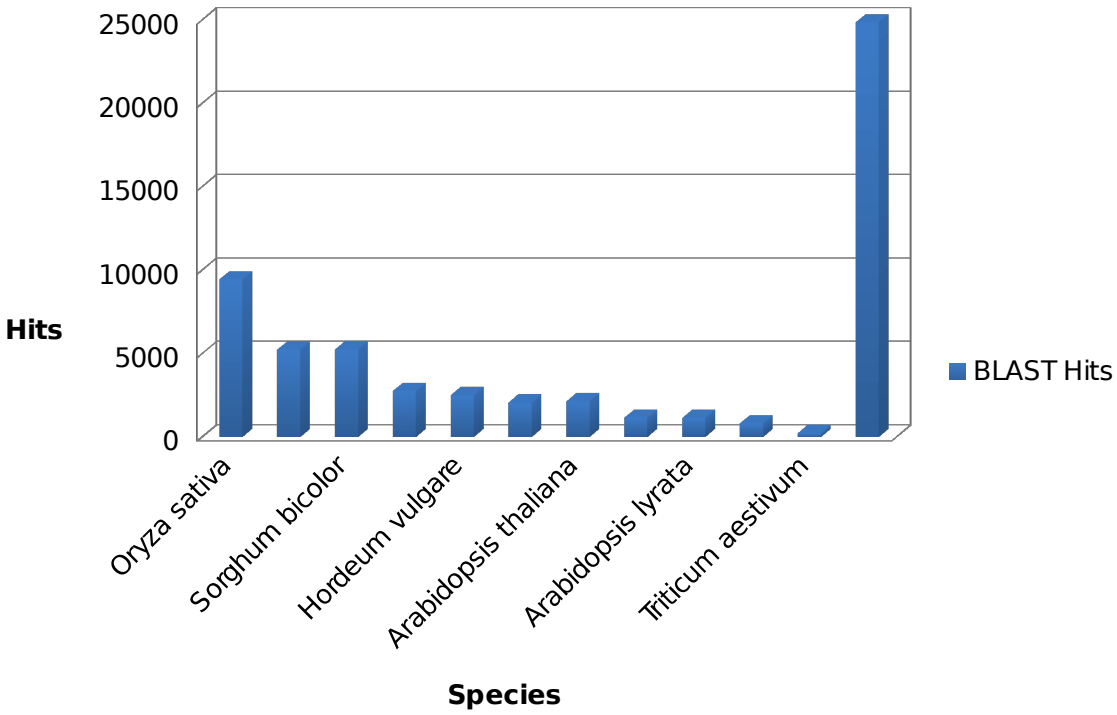


Fig 4.7 BLAST hit distribution of pearl millet unigenes with other crop species

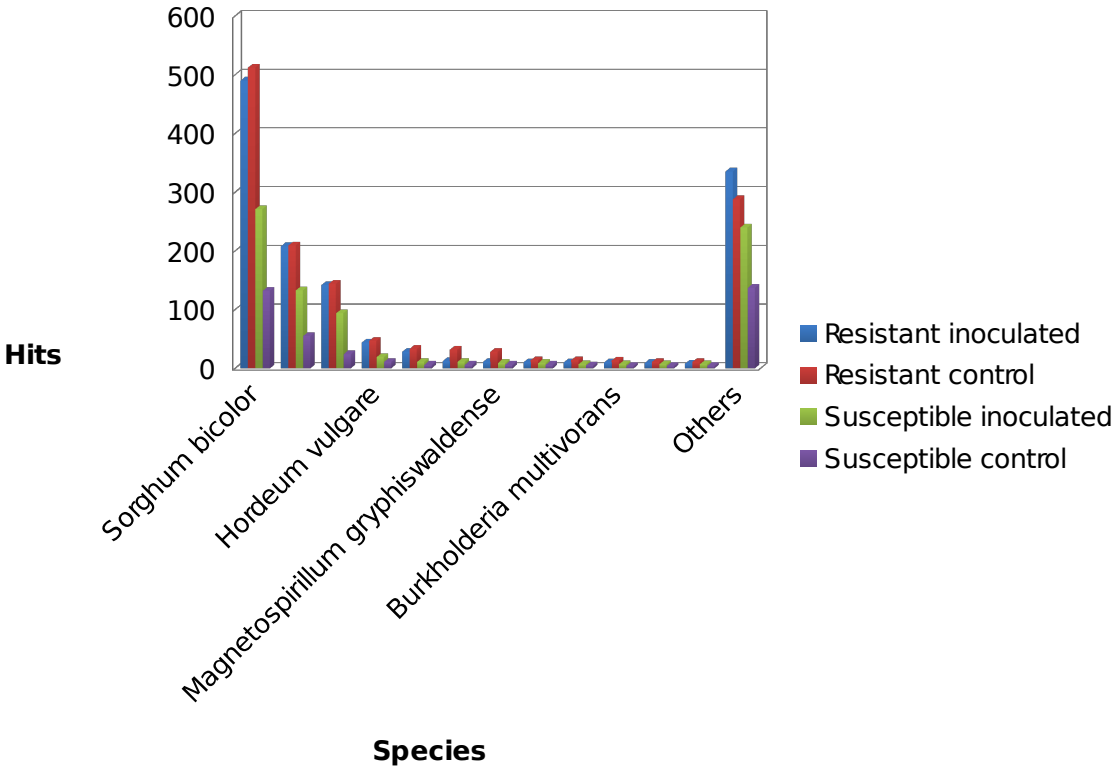


Fig 4.8 Top BLAST hits of pearl millet sequences with other species

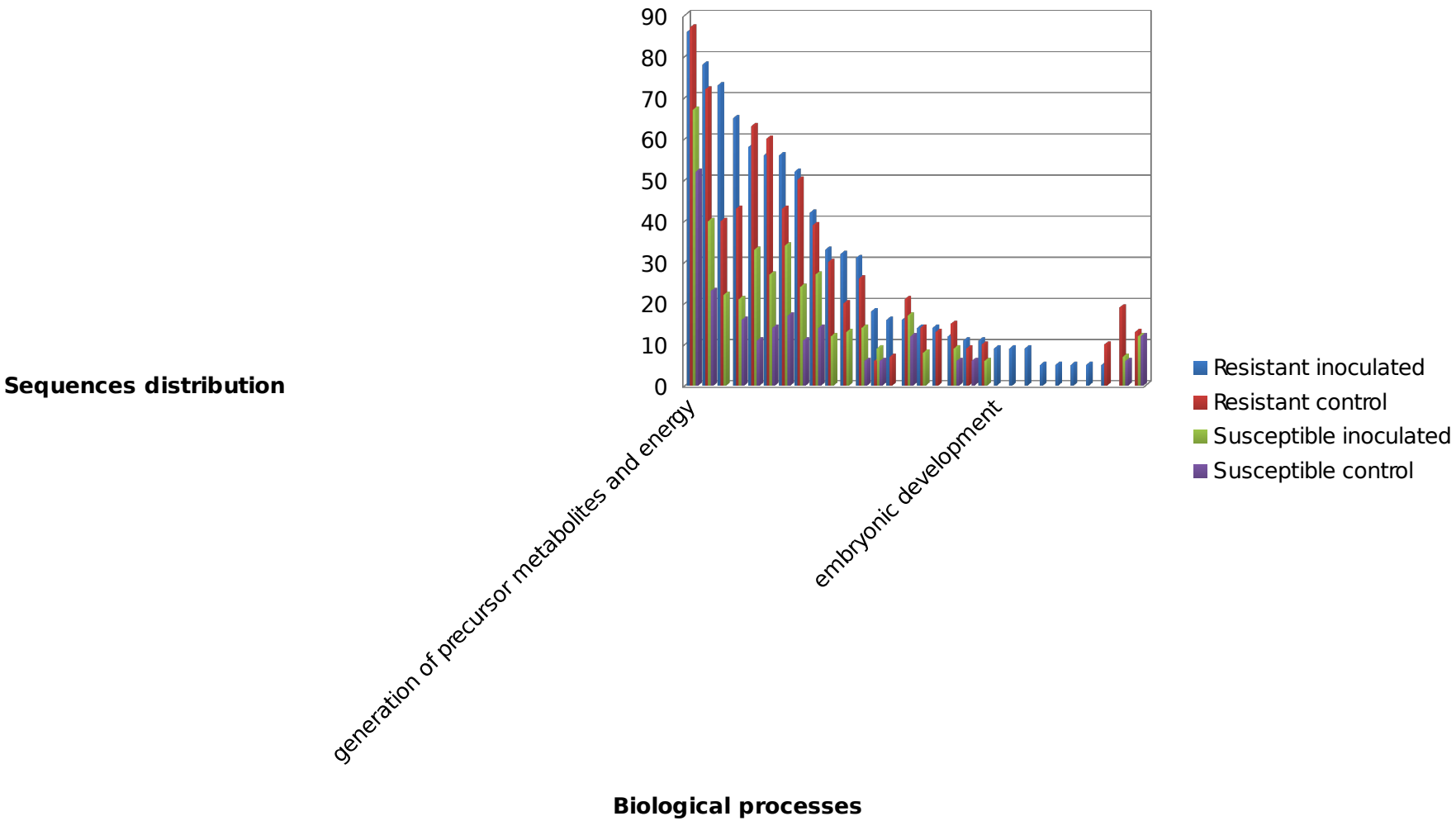


Fig 4.9 Depiction of Biological processes of pearl millet sequences

(Multi level GO graph with sequence cut off 5)

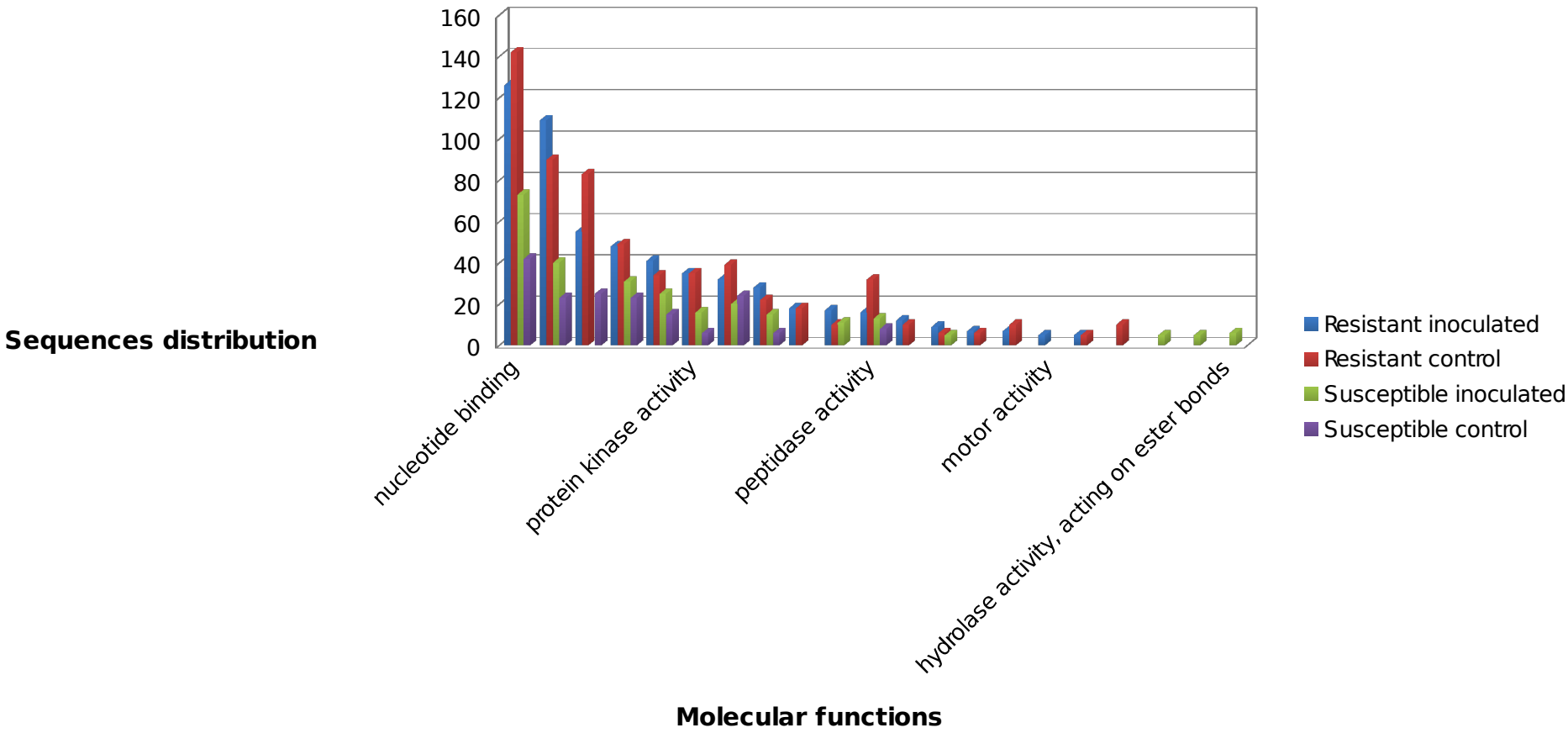


Fig 4.10 Depiction of Molecular processes of pearl millet sequences (Multi level GO graph with sequence cut off 5)

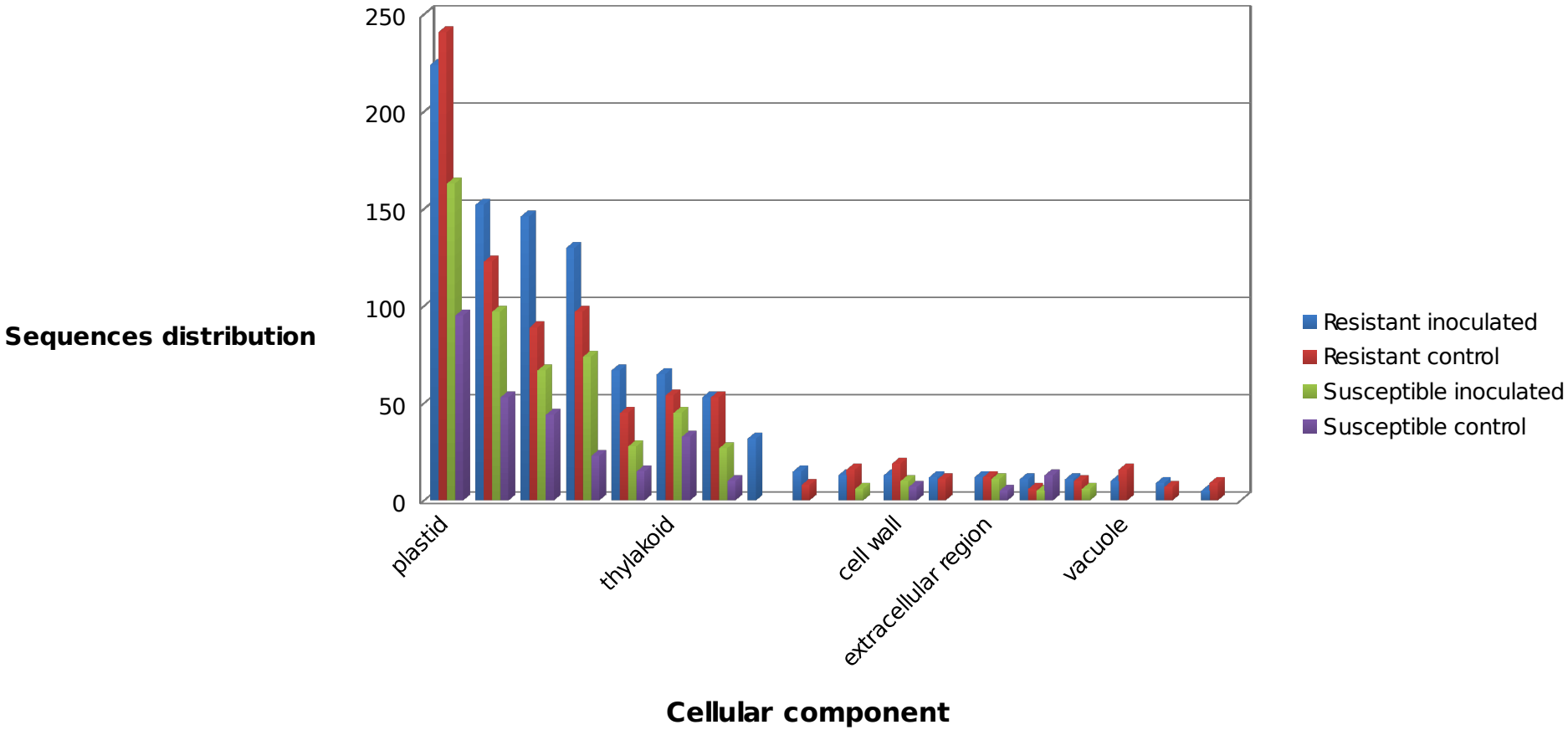
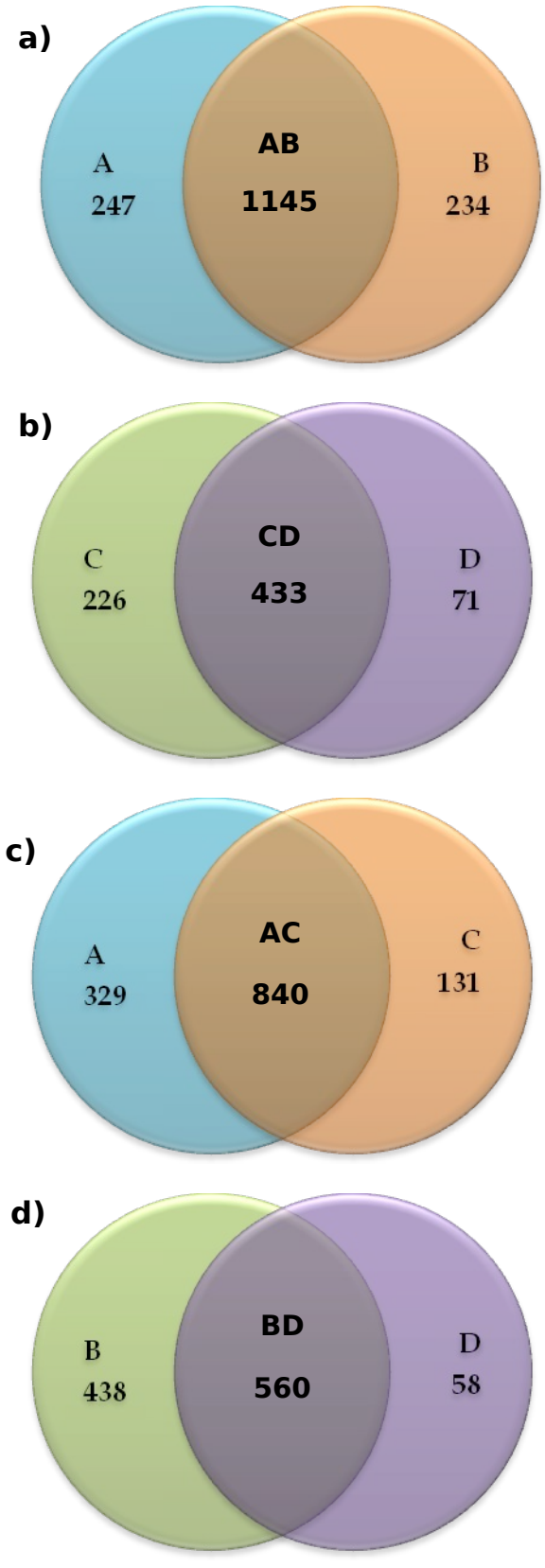


Fig 4.11 Depiction of Cellular processes of pearl millet sequences

(Multi level GO graph with sequence cut off 5)



A: Resistant inoculated unigenes

B: Resistant control unigenes

C: Susceptible inoculated unigenes

D: Susceptible control unigenes

Fig 4.12 (a-d) Venn diagrams showing differentially expressed unigenes for pearl millet genotypes

a) Resistant inoculated v/s Resistant control

b) Susceptible inoculated v/s susceptible control

c) Resistant inoculated v/s Susceptible inoculated

d) Resistant control v/s susceptible control

Plate I: Pearl millet leaves showing typical symptoms of downy mildew pathogen *Sclerospora graminicola*

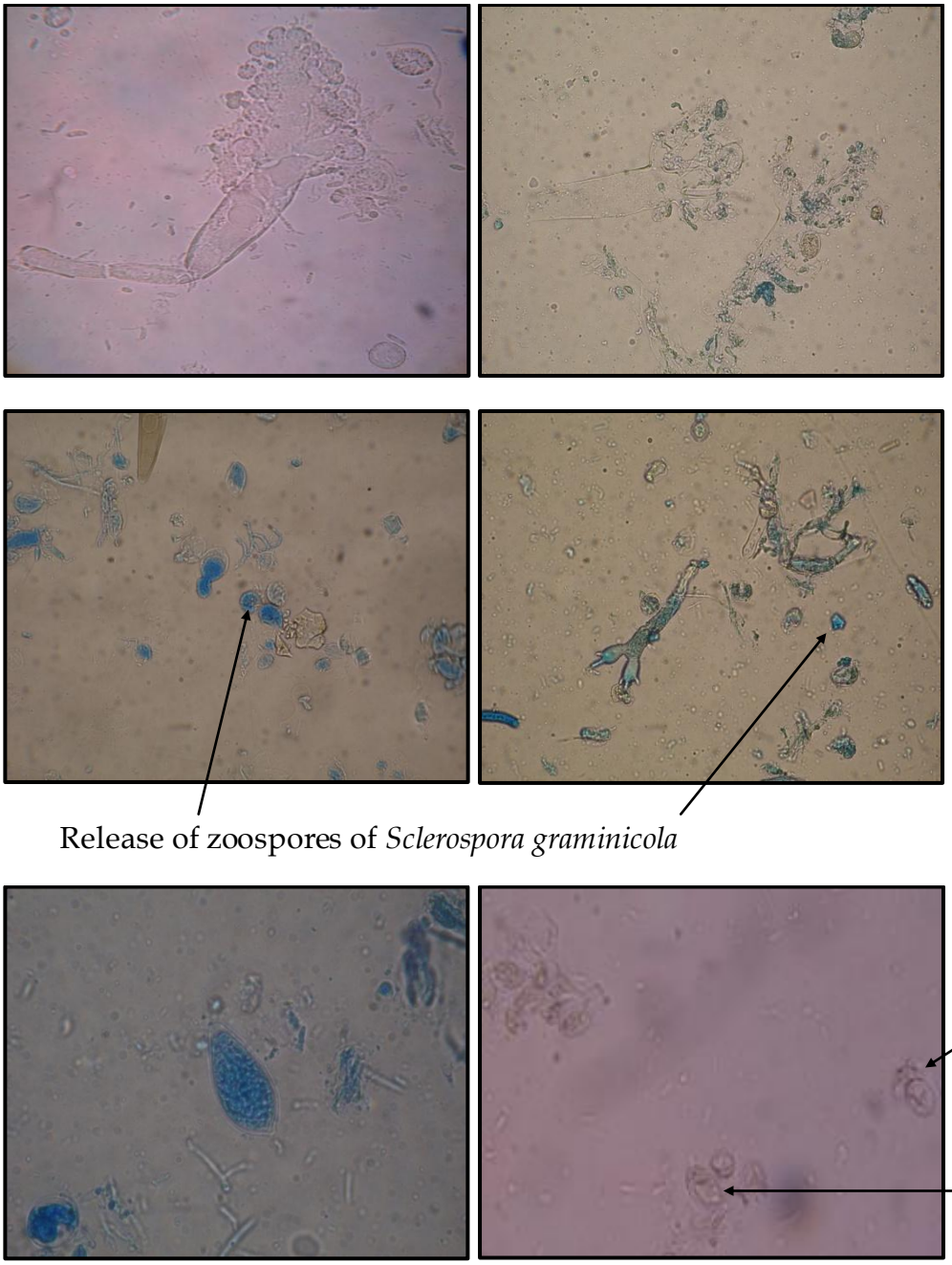
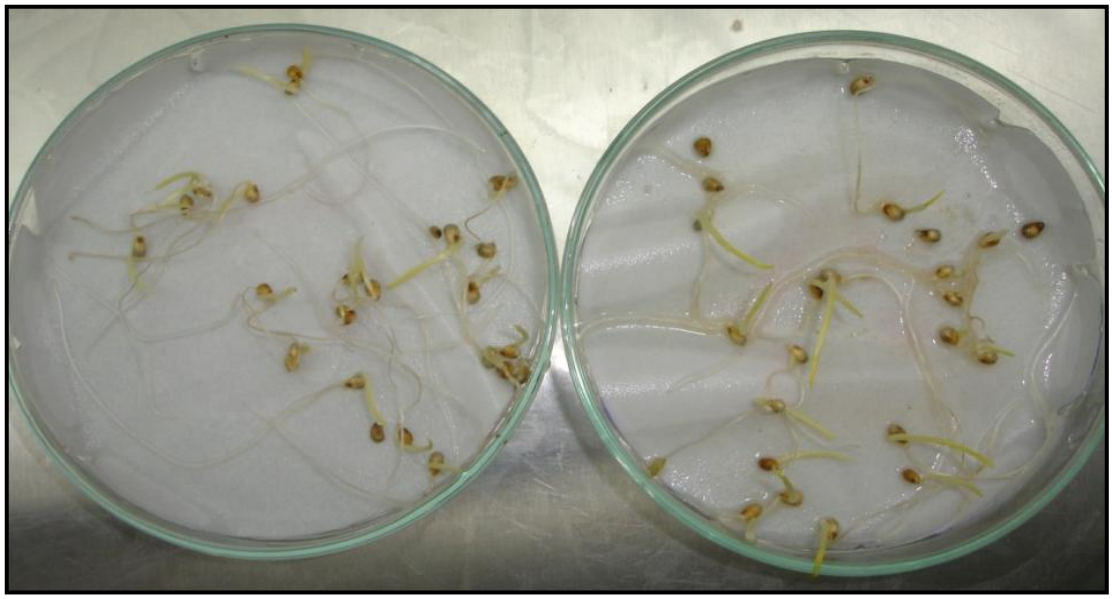


Plate II: Sporangium and palm shaped Sporangiophore of *Sclerospora graminicola* observed under microscope

a)



b)

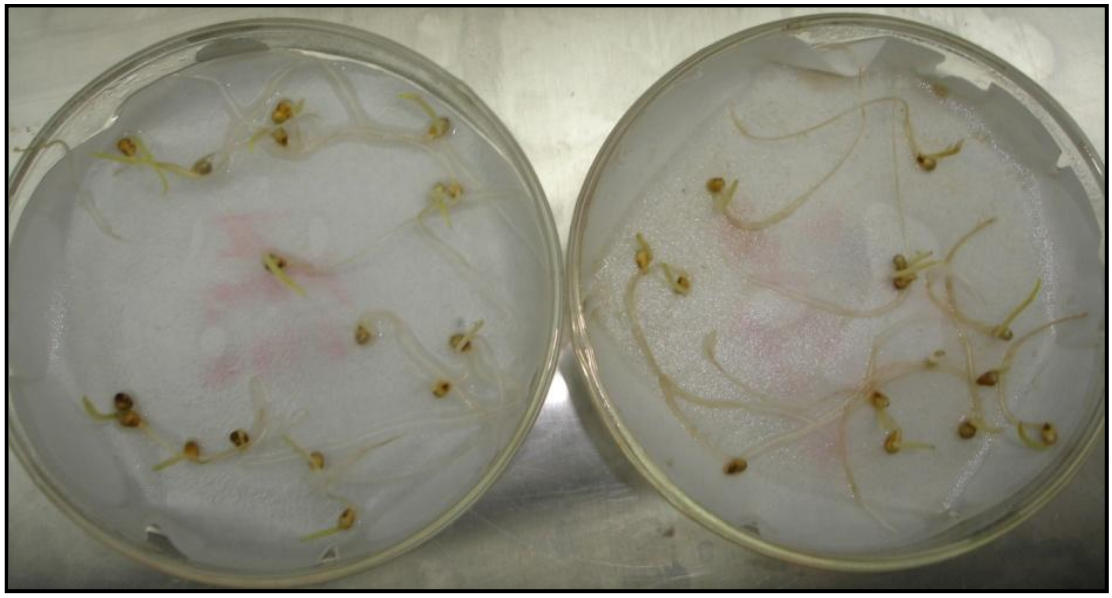
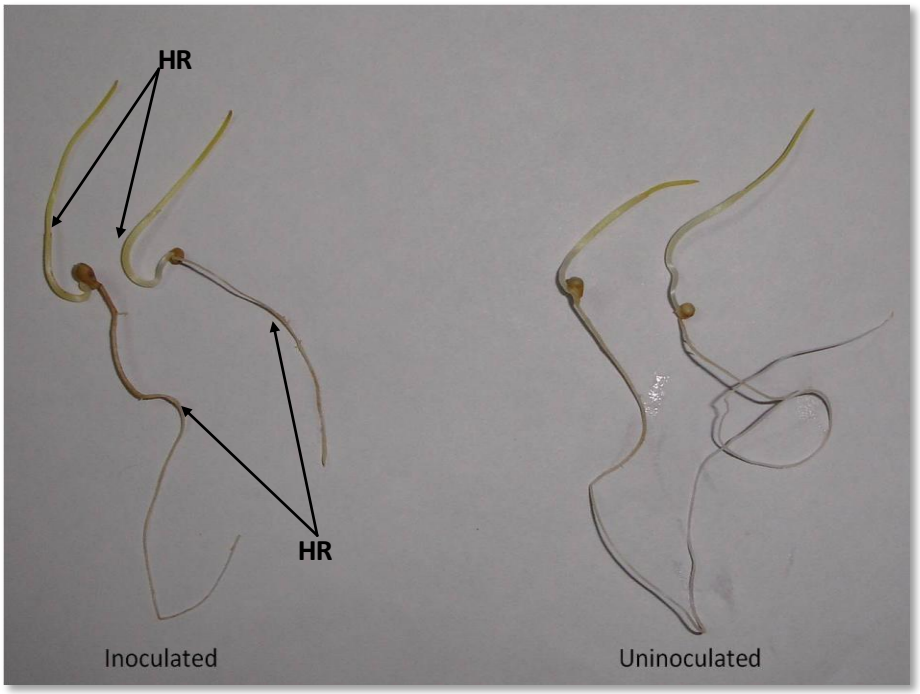


Plate III: Two day old grown seedlings of a) Resistant genotype J2290 and b) Susceptible genotype 7042S of pearl millet

a)



b)

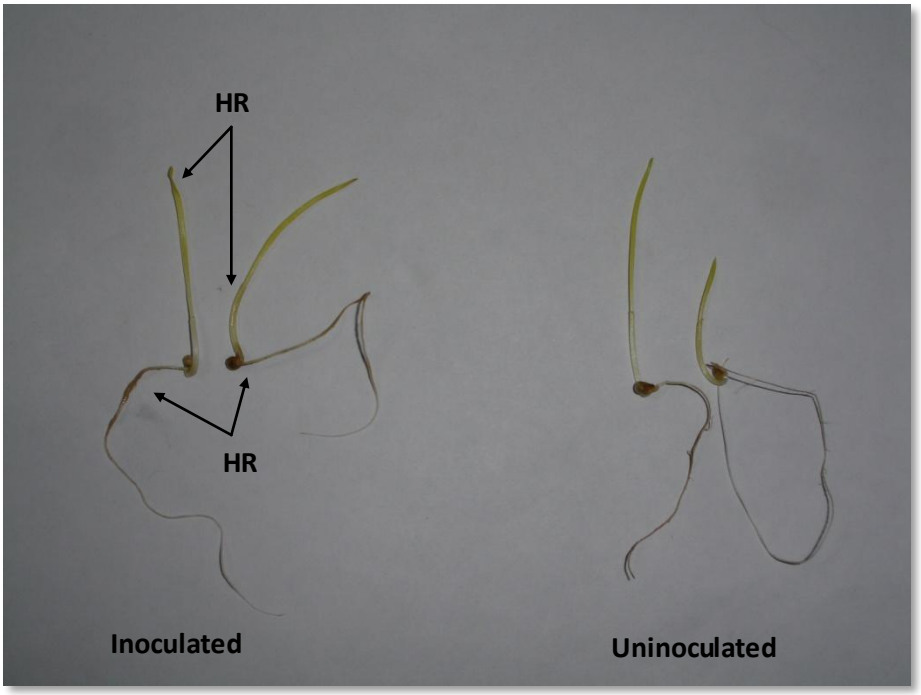


Plate IV: Seedlings showing hypersensitive response (HR) a) Resistant genotype J2290 and b) Susceptible genotype 7402S, 36 hpi with *Sclerospora graminicola* inoculum

Table 4.13 Candidate genes responsible for resistance in pearl millet against downy mildew pathogen

PR proteins	Enzymes	Resistance proteins	Transcription factors	Signaling
Peroxidase	Hydrolase	NBS LRR	WRKY25	MAP kinase
Chitinase	Acid phosphatase	*Rxo1*	DREB	Protein kinase
Glucanase	Glucosidase	*Mlo*	Creb	Serine/threonine
Thaumatin	C- 3-OMT	*Edr*	F- box	Shaggy kinase
Thionine	Chalcone synthase	Immediate early fungal elicitor protein	Zinc finger	DAG kinase
Ribonuclease	Isochorismate lyase	Snf related proteins	Myb	Ionositol polyphosphate 2 kinase
	SOD	*Avr9 cf* elicited protein	DEAD box	Aspartate kinase
	Xylanase	Rav like proteins	Fyve finger	Cbl interacting kinase
	Lipoxygenase	Tbc domain containing proteins	Ring finger family	
	PAL	Gsd motif lipase	G-Box	
	Catalase	Ankyrin repeat proteins		
	Esterase	Atp adp translocator		
	Allene oxide synthase	Phd finger type		
	Arginosuccinate synthase	Expansin		
	h^+ ATPase	Kelch repeat proteins		
	Glutathione S-transferase	two-component response regulator-like		

		prr95		